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(54) Title: VACCINES

(57) Abstract: The invention provides a nucleotide sequence that encodes an HIV-1 gag protein or fragment thereof containing a gag epitope and a second HIV antigen or a fragment encoding an epitope of said second HIV antigen, operably linked to a heterologous promoter. Preferred polynucleotide sequences further encodes nef or a fragment thereof and RT or a fragment thereof.

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Vaccines

Field of the Invention

The present invention relates to nucleic acid constructs, host cells comprising such constructs and their use in nucleic acid vaccines. The invention further relates to vaccine formulations comprising such constructs and the use of such formulations in medicine. The invention in particular relates to DNA vaccines that are useful in the prophylaxis and treatment of HIV infections, more particularly when administered by particle mediated delivery.

Background to the Invention

HIV-1 is the primary cause of the acquired immune deficiency syndrome (AIDS) which is regarded as one of the world's major health problems. Although extensive research throughout the world has been conducted to produce a vaccine, such efforts thus far have not been successful.

Non-envelope proteins of HIV-1 have been described and include for example internal structure proteins such as the products of the *gag* and *pol* genes and, other non-structural proteins such as Rev, Nef, Vif and Tat (Green et al., New England J. Med, **324**, 5, 308 et seq (1991) and Bryant et al. (Ed. Pizzo), Pediatr. Infect. Dis. J., **11**, 5, 390 et seq (1992).

The Gag gene is translated from the full-length RNA to yield a precursor polyprotein which is subsequently cleaved into 3 - 5 capsid proteins; the matrix protein, capsid protein and nucleic acid binding protein and

protease. (1. Fundamental Virology, Fields BN, Knipe DM and Howley M 1996 2. Fields Virology vol 2 1996).

The gag gene gives rise to the 55-kilodalton (kD) Gag precursor protein, also called p55, which is expressed from the unspliced viral mRNA. During translation, the N terminus of p55 is myristoylated, triggering its association with the cytoplasmic aspect of cell membranes. The membrane-associated Gag polyprotein recruits two copies of the viral genomic RNA along with other viral and cellular proteins that triggers the budding of the viral particle from the surface of an infected cell. After budding, p55 is cleaved by the virally encoded protease (a product of the pol gene) during the process of viral maturation into four smaller proteins designated MA (matrix [p17]), CA (capsid [p24]), NC (nucleocapsid [p9]), and p6.(4)

In addition to the 3 major Gag protein, all Gag precursors contain several other regions, which are cleaved out and remain in the virion as peptides of various sizes. These proteins have different roles e.g. the p2 protein has a proposed role in regulating activity of the protease and contributes to the correct timing of proteolytic processing.

The MA polypeptide is derived from the N-terminal, myristoylated end of p55. Most MA molecules remain attached to the inner surface of the virion lipid bilayer, stabilizing the particle. A subset of MA is recruited inside the deeper layers of the virion where it becomes part of the complex which escorts the viral DNA to the nucleus.(5) These MA molecules facilitate the nuclear transport of the viral genome because a karyophilic signal on MA is recognized by the cellular

nuclear import machinery. This phenomenon allows HIV to infect nondividing cells, an unusual property for a retrovirus.

The p24 (CA) protein forms the conical core of viral particles. Cyclophilin A has been demonstrated to interact with the p24 region of p55 leading to its incorporation into HIV particles. The interaction between Gag and cyclophilin A is essential because the disruption of this interaction by cyclosporine A inhibits viral replication.

The NC region of Gag is responsible for specifically recognizing the so-called packaging signal of HIV. The packaging signal consists of four stem loop structures located near the 5' end of the viral RNA, and is sufficient to mediate the incorporation of a heterologous RNA into HIV-1 virions. NC binds to the packaging signal through interactions mediated by two zinc-finger motifs. NC also facilitates reverse transcription.

The p6 polypeptide region mediates interactions between p55 Gag and the accessory protein Vpr, leading to the incorporation of Vpr into assembling virions. The p6 region also contains a so-called late domain which is required for the efficient release of budding virions from an infected cell

The Pol gene encodes two proteins containing the two activities needed by the virus in early infection, the RT and the integrase protein needed for integration of viral DNA into cell DNA. The primary product of Pol is cleaved by the virion protease to yield the amino terminal RT peptide which contains activities necessary for DNA synthesis (RNA and DNA directed DNA polymerase, ribouclease H) and carboxy terminal integrase protein.

HIV RT is a heterodimer of full-length RT (p66) and a cleavage product (p51) lacking the carboxy terminal Rnase integrase domain.

RT is one of the most highly conserved proteins encoded by the retroviral genome. Two major activities of RT are the DNA Pol and Ribonuclease H. The DNA Pol activity of RT uses RNA and DNA as templates interchangeably and like all DNA polymerases known is unable to initiate DNA synthesis de novo, but requires a pre existing molecule to serve as a primer (RNA).

The Rnase H activity inherent in all RT proteins plays the essential role early in replication of removing the RNA genome as DNA synthesis proceeds. It selectively degrades the RNA from all RNA - DNA hybrid molecules. Structurally the polymerase and ribo H occupy separate, non-overlapping domains with the Pol covering the amino two thirds of the Pol.

The p66 catalytic subunit is folded into 5 distinct subdomains. The amino terminal 23 of these have the portion with RT activity. Carboxy term to these is the Rnase H Domain.

After infection of the host cell, the retroviral RNA genome is copied into linear ds DNA by the reverse transcriptase that is present in the infecting particle. The integrase (reviewed in Skalka AM '99 Adv in Virus Res 52 271-273) recognises the ends of the viral DNA, trims them and accompanies the viral DNA to a host chromosomal site to catalyse integration. Many sites in the host DNA can be targets for integration. Although the integrase is sufficient to catalyse integration in vitro, it is not the only protein associated with the viral DNA in vivo -

the large protein - viral DNA complex isolated from the infected cells has been denoted the pre integration complex. This facilitates the acquisition of the host cell genes by progeny viral genomes.

The integrase is made up of 3 distinct domains, the N terminal domain, the catalytic core and the c terjminal domain. The catalytic core domain contains all of the requirements for the chemistry of polynucleotidyl transfer.

The Nef protein is known to cause the removal of CD4, the HIV receptor, from the cell surface, but the biological importance of this function is debated. Additionally Nef interacts with the signal pathway of T cells and induces an active state, which in turn may promote more efficient gene expression. Some HIV isolates have mutations in this region, which cause them not to encode functional protein and are severely compromised in their replication and pathogenesis in vivo.

DNA vaccines usually consist of a bacterial plasmid vector into which is inserted a strong promoter, the gene of interest which encodes for an antigenic peptide and a polyadenylation/transcriptional termination sequences. The gene of interest may encode a full protein or simply an antigenic peptide sequence relating to the pathogen, tumour or other agent which is intended to be protected against. The plasmid can be grown in bacteria, such as for example E.coli and then isolated and prepared in an appropriate medium, depending upon the intended route of administration, before being administered to the host. Following administration the plasmid is taken up by cells of the host where the encoded peptide is produced. The plasmid vector will preferably be made without an origin

of replication which is functional in eukaryotic cells, in order to prevent plasmid replication in the mammalian host and integration within chromosomal DNA of the animal concerned.

There are a number of advantages of DNA vaccination relative to traditional vaccination techniques. First, it is predicted that because of the proteins which are encoded by the DNA sequence are synthesised in the host, the structure or conformation of the protein will be similar to the native protein associated with the disease state. It is also likely that DNA vaccination will offer protection against different strains of a virus, by generating cytotoxic T lymphcyte response that recognise epitopes from conserved proteins. Furthermore, because the plasmids are taken up by the host cells where antigenic protein can be produced, a long-lasting immune response will be elicited. The technology also offers the possibility of combing diverse immunogens into a single preparation to facilitate simultaneous immunisation in relation to a number of disease states.

Helpful background information in relation to DNA vaccination is provided in Donnelly et al "DNA vaccines" Ann. Rev Immunol. 1997 15: 617-648, the disclosure of which is included herein in its entirety by way of reference.

Summary of the Invention

The present invention provides novel constructs for use in nucleic acid vaccines for the prophylaxis and treatment of HIV infections and AIDS.

Accordingly, in a first aspect, there is provided a nucleic acid molecule comprising a nucleotide sequence encoding HIV gag protein or fragment thereof linked to a nucleotide sequence encoding a further HIV antigen or fragment thereof and operably linked to a heterologous promoter. The fragment of said nucleotide sequence will encode an HIV epitope and typically encode a peptide of at least 8 amino acids. The nucleotide sequence is preferably a DNA sequence and is preferably contained within a plasmid without an origin of replication. Such nucleic acid molecules are formulated with pharmaceutically acceptable excipient, carriers, diluents or adjuvants to produce pharmaceutical composition suitable for the treatment and/or prophylaxis of HIV infection and AIDS.

In a preferred embodiment the DNA sequence is formulated onto the surface of inert particles or beads suitable for particle mediated drug delivery. Preferably the beads are gold.

In a preferred embodiment of the invention there is provided a DNA sequence that highly expressed codes for gag protein which sequence is optimised to resemble the codon usage of genes in mammalian cells. In particular, the gag protein is optimised to resemble that of highly expressed human genes.

The DNA code has 4 letters (A, T, C and G) and uses these to spell three letter "codons" which represent the amino acids the proteins encoded in an organism's genes. The linear sequence of codons along the DNA molecule is translated into the linear sequence of amino acids in the protein(s) encoded by those genes. The code is highly degenerate, with 61 codons coding for the 20 natural

amino acids and 3 codons representing "stop" signals. Thus, most amino acids are coded for by more than one codon - in fact several are coded for by four or more different codons.

Where more than one codon is available to code for a given amino acid, it has been observed that the codon usage patterns of organisms are highly non-random. Different species show a different bias in their codon selection and, furthermore, utilisation of codons may be markedly different in a single species between genes which are expressed at high and low levels. This bias is different in viruses, plants, bacteria and mammalian cells, and some species show a stronger bias away from a random codon selection than others. For example, humans and other mammals are less strongly biased than certain bacteria or viruses. For these reasons, there is a significant probability that a mammalian gene expressed in E.coli or a foreign or recombinant gene expressed in mammalian cells will have an inappropriate distribution of codons for efficient expression. It is believed that the presence in a heterologous DNA sequence of clusters of codons or an abundance of codons which are rarely observed in the host in which expression is to occur, is predictive of low heterologous expression levels in that host.

In an embodiment of the present invention provides a gag polynucleotide sequence which encodes an amino acid sequence, wherein the codon usage pattern of the polynucleotide sequence resembles that of highly expressed mammalian genes. Preferably the polynucleotide sequence is a DNA sequence. Desirably the codon usage pattern of the polynucleotide sequence is typical of highly expressed human genes.

In the polynucleotides of the present invention, the codon usage pattern is altered from that typical of human immunodeficiency viruses to more closely represent the codon bias of the target organism, e.g. a mammal, especially a human. The "codon usage coefficient" is a measure of how closely the codon pattern of a given polynucleotide sequence resembles that of a target species. Codon frequencies can be derived from literature sources for the highly expressed genes of many species (see e.g. Nakamura et.al. Nucleic Acids Research 1996, **24**:214-215). The codon frequencies for each of the 61 codons (expressed as the number of occurrences occurrence per 1000 codons of the selected class of genes) are normalised for each of the twenty natural amino acids, so that the value for the most frequently used codon for each amino acid is set to 1 and the frequencies for the less common codons are scaled to lie between zero and 1. Thus each of the 61 codons is assigned a value of 1 or lower for the highly expressed genes of the target species. In order to calculate a codon usage coefficient for a specific polynucleotide, relative to the highly expressed genes of that species, the scaled value for each codon of the specific polynucleotide are noted and the geometric mean of all these values is taken (by dividing the sum of the natural logs of these values by the total number of codons and take the anti-log). The coefficient will have a value between zero and 1 and the higher the coefficient the more codons in the polynucleotide are frequently used codons. If a polynucleotide sequence has a codon usage coefficient of 1, all of the codons are "most frequent" codons for highly expressed genes of the target species.

According to the present invention, the codon usage pattern of the polynucleotide will preferably exclude codons with an RSCU value of less than 0.2 in highly expressed genes of the target organism. Alternatively, the codon usage pattern will exclude codons representing <10% of the codons used for a particular amino acid. A relative synonymous codon usage (RSCU) value is the observed number of codons divided by the number expected if all codons for that amino acid were used equally frequently. A polynucleotide of the present invention will generally have a codon usage coefficient (or RSCU) for highly expressed human genes of greater than 0.3, preferably greater than 0.4, most preferably greater than 0.5 Codon usage tables for human can also be found in Genebank.

In comparison, a highly expressed beta actin gene has a RSCU of 0.747. The codon usage table for a homo sapiens is set out below:

Codon Usage Table 1:

***Homo sapiens* [gbpri]: 27143 CDS's (12816923 codons)**

fields: [triplet] [frequency: **per thousand**] ([number])

UUU 17.0(217684)	UCU 14.8(189419)	UAU 12.1(155645)	UGU 10.0(127719)
UUC 20.5(262753)	UCC 17.5(224470)	UAC 15.8(202481)	UGC 12.3(157257)
UUA 7.3(93924)	UCA 11.9(152074)	UAA 0.7(9195)	UGA 1.3(16025)
UUG 12.5(159611)	UCG 4.5(57572)	UAG 0.5(6789)	UGG 12.9(165930)
CUU 12.8(163707)	CCU 17.3(222146)	CAU 10.5(134186)	CGU 4.6(59454)
CUC 19.3(247391)	CCC 20.0(256235)	CAC 14.9(190928)	CGC 10.8(137865)
CUA 7.0(89078)	CCA 16.7(214583)	CAA 12.0(153590)	CGA 6.3(80709)
CUG 39.7(509096)	CCG 7.0(89619)	CAG 34.5(441727)	CGG 11.6(148666)
AUU 15.8(202844)	ACU 12.9(165392)	AAU 17.0(218508)	AGU 12.0(154442)
AUC 21.6(277066)	ACC 19.3(247805)	AAC 19.8(253475)	AGC 19.3(247583)
AUA 7.2(92133)	ACA 14.9(191518)	AAA 24.0(308123)	AGA 11.5(147264)
AUG 22.3(285776)	ACG 6.3(80369)	AAG 32.6(418141)	AGG 11.3(145276)

GUU 10.9(139611) GCU 18.5(236639) GAU 22.4(286742) GGU 10.8(138606)
GUC 14.6(187333) GCC 28.3(362086) GAC 26.1(334158) GGC 22.7(290904)
GUA 7.0(89644) GCA 15.9(203310) GAA 29.1(373151) GGA 16.4(210643)
GUG 28.8(369006) GCG 7.5(96455) GAG 40.2(515485) GGG 16.4(209907)

Coding GC 52.51% 1st letter GC 56.04% 2nd letter GC 42.35% 3rd letter GC 59.13%

Codon Usage Table 2 (preferred):

Codon usage for human (highly expressed) genes 1/24/91 (human_high.cod)

AmAcid	Codon	Number	/1000	Fraction	..
Gly	GGG	905.00	18.76	0.24	
Gly	GGA	525.00	10.88	0.14	
Gly	GGT	441.00	9.14	0.12	
Gly	GGC	1867.00	38.70	0.50	
Glu	GAG	2420.00	50.16	0.75	
Glu	GAA	792.00	16.42	0.25	
Asp	GAT	592.00	12.27	0.25	
Asp	GAC	1821.00	37.75	0.75	
Val	GTG	1866.00	38.68	0.64	
Val	GTA	134.00	2.78	0.05	
Val	GTT	198.00	4.10	0.07	
Val	GTC	728.00	15.09	0.25	
Ala	GCG	652.00	13.51	0.17	
Ala	GCA	488.00	10.12	0.13	
Ala	GCT	654.00	13.56	0.17	
Ala	GCC	2057.00	42.64	0.53	
Arg	AGG	512.00	10.61	0.18	
Arg	AGA	298.00	6.18	0.10	
Ser	AGT	354.00	7.34	0.10	
Ser	AGC	1171.00	24.27	0.34	
Lys	AAG	2117.00	43.88	0.82	
Lys	AAA	471.00	9.76	0.18	
Asn	AAT	314.00	6.51	0.22	
Asn	AAC	1120.00	23.22	0.78	
Met	ATG	1077.00	22.32	1.00	
Ile	ATA	88.00	1.82	0.05	
Ile	ATT	315.00	6.53	0.18	
Ile	ATC	1369.00	28.38	0.77	
Thr	ACG	405.00	8.40	0.15	

Thr	ACA	373.00	7.73	0.14
Thr	ACT	358.00	7.42	0.14
Thr	ACC	1502.00	31.13	0.57
Trp	TGG	652.00	13.51	1.00
End	TGA	109.00	2.26	0.55
Cys	TGT	325.00	6.74	0.32
Cys	TGC	706.00	14.63	0.68
End	TAG	42.00	0.87	0.21
End	TAA	46.00	0.95	0.23
Tyr	TAT	360.00	7.46	0.26
Tyr	TAC	1042.00	21.60	0.74
Leu	TTG	313.00	6.49	0.06
Leu	TTA	76.00	1.58	0.02
Phe	TTT	336.00	6.96	0.20
Phe	TTC	1377.00	28.54	0.80
Ser	TCG	325.00	6.74	0.09
Ser	TCA	165.00	3.42	0.05
Ser	TCT	450.00	9.33	0.13
Ser	TCC	958.00	19.86	0.28
Arg	CGG	611.00	12.67	0.21
Arg	CGA	183.00	3.79	0.06
Arg	CGT	210.00	4.35	0.07
Arg	CGC	1086.00	22.51	0.37
Gln	CAG	2020.00	41.87	0.88
Gln	CAA	283.00	5.87	0.12
His	CAT	234.00	4.85	0.21
His	CAC	870.00	18.03	0.79
Leu	CTG	2884.00	59.78	0.58
Leu	CTA	166.00	3.44	0.03
Leu	CTT	238.00	4.93	0.05
Leu	CTC	1276.00	26.45	0.26
Pro	CCG	482.00	9.99	0.17
Pro	CCA	456.00	9.45	0.16
Pro	CCT	568.00	11.77	0.19
Pro	CCC	1410.00	29.23	0.48

According to a further aspect of the invention, an expression vector is provided which comprises and is capable of directing the expression of a polynucleotide sequence according to the first aspect of the invention, in particular the codon usage pattern of the gag

polynucleotide sequence is typical of highly expressed mammalian genes, preferably highly expressed human genes. The vector may be suitable for driving expression of heterologous DNA in bacterial insect or mammalian cells, particularly human cells. In one embodiment, the expression vector is p7313 (see figure 1).

In a third embodiment there is provided a gag gene under the control of a heterologous promoter fused to a DNA sequence encoding NEF, a fragment thereof, or HIV Reverse Transcriptase (RT) or fragment thereof. The gag portion of the gene may be either the N or C terminal portion of the fusion.

In a preferred embodiment, the gag gene does not encode the gag p6 peptide. Preferably the NEF gene is truncated to remove the sequence encoding the N terminal region i.e. removal of 30-85, preferably 60-85, typically about 81, preferably the N terminal 65 amino acids.

In a further embodiment the RT gene is also optimised to resemble a highly expressed human gene. The RT preferably encodes a mutation to substantially inactivate any reverse transcriptase activity. A preferred inactivation mutation involves the substitution of W tryptophan 229 for K (lysine).

According to a further aspect of the invention, a host cell comprising a polynucleotide sequence according to the invention, or an expression vector according to the invention is provided. The host cell may be bacterial, e.g. E.coli, mammalian, e.g. human, or may be an insect cell. Mammalian cells comprising a vector according to the present invention may be cultured cells transfected

in vitro or may be transfected in vivo by administration of the vector to the mammal.

The present invention further provides a pharmaceutical composition comprising a polynucleotide sequence according to the invention. Preferably the composition comprises a DNA vector. In preferred embodiments the composition comprises a plurality of particles, preferably gold particles, coated with DNA comprising a vector encoding a polynucleotide sequence of the invention. Preferably the sequence encodes an HIV gag amino acid sequence, wherein the codon usage pattern of the polynucleotide sequence is typical of highly expressed mammalian genes, particularly human genes. In alternative embodiments, the composition comprises a pharmaceutically acceptable excipient and a DNA vector according to the second aspect of the present invention. The composition may also include an adjuvant.

Thus it is an embodiment of the invention that the vectors of the invention be utilised with immunostimulatory agents. Preferably the immunostimulatory agent are administered at the same time as the nucleic acid vector of the invention and in preferred embodiments are formulated together. Such immunostimulatory agents include, but this list is by no means exhaustive and does not preclude other agents: synthetic imidazoquinolines such as imiquimod [S-26308, R-837], (Harrison, et al. 'Reduction of recurrent HSV disease using imiquimod alone or combined with a glycoprotein vaccine', Vaccine 19: 1820-1826, (2001)); and resiquimod [S-28463, R-848] (Vasilakos, et al. ' Adjuvant activites of immune response modifier R-848: Comparison with CpG ODN', Cellular immunology 204: 64-74 (2000).), Schiff bases of carbonyls and amines that are

constitutively expressed on antigen presenting cell and T-cell surfaces, such as tucaresol (Rhodes, J. et al. ' Therapeutic potentiation of the immune system by costimulatory Schiff-base-forming drugs', Nature 377: 71-75 (1995)), cytokine, chemokine and co-stimulatory molecules as either protein or peptide, this would include pro-inflammatory cytokines such as GM-CSF, IL-1 alpha, IL-1 beta, TGF- alpha and TGF - beta, Th1 inducers such as interferon gamma, IL-2, IL-12, IL-15 and IL-18, Th2 inducers such as IL-4, IL-5, IL-6, IL-10 and IL-13 and other chemokine and co-stimulatory genes such as MCP-1, MIP-1 alpha, MIP-1 beta, RANTES, TCA-3, CD80, CD86 and CD40L, , other immunostimulatory targeting ligands such as CTLA-4 and L-selectin, apoptosis stimulating proteins and peptides such as Fas, (49), synthetic lipid based adjuvants, such as vaxfectin, (Reyes et al., 'Vaxfectin enhances antigen specific antibody titres and maintains Th1 type immune responses to plasmid DNA immunization', Vaccine 19: 3778-3786) squalene, alpha- tocopherol, polysorbate 80, DOPC and cholesterol, endotoxin, [LPS], Beutler, B., 'Endotoxin, 'Toll-like receptor 4, and the afferent limb of innate immunity', Current Opinion in Microbiology 3: 23-30 (2000)) ; CpG oligo- and di-nucleotides, Sato, Y. et al., 'Immunostimulatory DNA sequences necessary for effective intradermal gene immunization', Science 273 (5273): 352-354 (1996). Hemmi, H. et al., 'A Toll-like receptor recognizes bacterial DNA', Nature 408: 740-745, (2000) and other potential ligands that trigger Toll receptors to produce Th1-inducing cytokines, such as synthetic Mycobacterial lipoproteins, Mycobacterial protein p19, peptidoglycan, teichoic acid and lipid A.

Certain preferred adjuvants for eliciting a predominantly Th1-type response include, for example, a Lipid A

derivative such as monophosphoryl lipid A, or preferably 3-de-O-acylated monophosphoryl lipid A. MPL® adjuvants are available from Corixa Corporation (Seattle, WA; *see*, for example, US Patent Nos. 4,436,727; 4,877,611; 4,866,034 and 4,912,094). CpG-containing oligonucleotides (in which the CpG dinucleotide is unmethylated) also induce a predominantly Th1 response. Such oligonucleotides are well known and are described, for example, in WO 96/02555, WO 99/33488 and U.S. Patent Nos. 6,008,200 and 5,856,462. Immunostimulatory DNA sequences are also described, for example, by Sato et al., *Science* 273:352, 1996. Another preferred adjuvant comprises a saponin, such as Quil A, or derivatives thereof, including QS21 and QS7 (Aquila Biopharmaceuticals Inc., Framingham, MA); Escin; Digitonin; or *Gypsophila* or *Chenopodium quinoa* saponins.

Also provided are the use of a polynucleotide according to the invention, or of a vector according to the invention, in the treatment or prophylaxis of an HIV infection.

The present invention also provides methods of treating or preventing HIV infections, any symptoms or diseases associated therewith, comprising administering an effective amount of a polynucleotide, a vector or a pharmaceutical composition according to the invention. Administration of a pharmaceutical composition may take the form of one or more individual doses, for example as repeat doses of the same DNA plasmid, or in a "prime-boost" therapeutic vaccination regime. In certain cases the "prime" vaccination may be via particle mediated DNA delivery of a polynucleotide according to the present invention, preferably incorporated into a plasmid-derived vector and the "boost" by administration of a recombinant

viral vector comprising the same polynucleotide sequence, or boosting with the protein in adjuvant. Conversly the priming may be with the viral vector or with a protein formulation typically a protein formulated in adjuvant and the boost a DNA vaccine of the present invention. Multiple doses of prime and/or boost may be employed.

In embodiments of the invention fragments of gag, nef or RT proteins are contemplated. For example, a polynucleotide of the invention may encode a fragment of an HIV gag, nef or RT protein. A polynucleotide which encodes a fragment of at least 8, for example 8-10 amino acids or up to 20, 50, 60, 70, 80, 100, 150 or 200 amino acids in length is considered to fall within the scope of the invention as long as the encoded oligo or polypeptide demonstrates HIV antigenicity. In particular, but not exclusively, this aspect of the invention encompasses the situation when the polynucleotide encodes a fragment of a complete HIV protein sequence and may represent one or more discrete epitopes of that protein. Such fragments may be codon optimised such that the fragment has a codon usage pattern which resembles that of a highly expressed mammalian gene.

Preferred constructs according to the present invention include:

1. p17, p24, fused to truncated NEF (devoid of nucleotides encoding terminal amino-acids 1-65)

2. p17, p24, RT, truncated NEF (devoid of nucleotides encoding terminal amino-acids 1-65)

3. p17, p24 (optimised gag) truncated NEF (devoid of nucleotides encoding terminal amino-acids 1-65)

4. p17, p24 (optimised gag) RT (optimised) truncated NEF (devoid of nucleotides encoding terminal amino-acids 1-85)

5. p17, p24, RT (optimised) truncated NEF (devoid of nucleotides encoding terminal amino-acids 1-65)

6. Truncated NEF - (devoid of nucleotide 1-65) fused to optimised p17, p24 gag.

7. Particularly preferred constructs of the invention include triple fusions RT-NEF-Gag, and RT-Gag-Nef particularly:

8. Optimised RT, truncated NEF and optimised P17, p24 (gag) (RNG)

and

9. Optimised RT, optimised p17, 24 (gag), Nef truncate (devoid of aa 1-65)RGN

It is preferred that the HIV constructs are derived from an HIV Clade B or Clade C, particularly clade B.

As discussed above, the present invention includes expression vectors that comprise the nucleotide sequences of the invention. Such expression vectors are routinely constructed in the art of molecular biology and may for example involve the use of plasmid DNA and appropriate initiators, promoters, enhancers and other elements, such as for example polyadenylation signals which may be necessary, and which are positioned in the correct orientation, in order to allow for protein expression. Other suitable vectors would be apparent to persons skilled in the art. By way of further example in this

regard we refer to Sambrook *et al*. Molecular Cloning: a Laboratory Manual. 2nd Edition. CSH Laboratory Press. (1989).

Preferably, a polynucleotide of the invention, or for use in the invention in a vector, is operably linked to a control sequence which is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence, such as a promoter, "operably linked" to a coding sequence is positioned in such a way that expression of the coding sequence is achieved under conditions compatible with the regulatory sequence.

The vectors may be, for example, plasmids, artificial chromosomes (e.g. BAC, PAC, YAC), virus or phage vectors provided with a origin of replication, optionally a promoter for the expression of the polynucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes, for example an ampicillin or kanamycin resistance gene in the case of a bacterial plasmid or a resistance gene for a fungal vector. Vectors may be used *in vitro*, for example for the production of DNA or RNA or used to transfect or transform a host cell, for example, a mammalian host cell e.g. for the production of protein encoded by the vector. The vectors may also be adapted to be used *in vivo*, for example in a method of DNA vaccination or of gene therapy.

Promoters and other expression regulation signals may be selected to be compatible with the host cell for which

expression is designed. For example, mammalian promoters include the metallothionein promoter, which can be induced in response to heavy metals such as cadmium, and the β-actin promoter. Viral promoters such as the SV40 large T antigen promoter, human cytomegalovirus (CMV) immediate early (IE) promoter, rous sarcoma virus LTR promoter, adenovirus promoter, or a HPV promoter, particularly the HPV upstream regulatory region (URR) may also be used. All these promoters are well described and readily available in the art.

A preferred promoter element is the CMV immediate early promoter, devoid of intron A but including exon 1. The promoter element may be the minimal promoter element or the enhanced promoter, the enhanced promoter being preferred. Accordingly there is provided a vector comprising a polynucleotide of the invention under the control of HCMV IE early promoter.

Examples of suitable viral vectors include herpes simplex viral vectors, vaccinia or alpha-virus vectors and retroviruses, including lentiviruses, adenoviruses and adeno-associated viruses. Gene transfer techniques using these viruses are known to those skilled in the art. Retrovirus vectors for example may be used to stably integrate the polynucleotide of the invention into the host genome, although such recombination is not preferred. Replication-defective adenovirus vectors by contrast remain episomal and therefore allow transient expression. Vectors capable of driving expression in insect cells (for example baculovirus vectors), in human cells, in yeast or in bacteria may be employed in order to produce quantities of the HIV protein encoded by the polynucleotides of the present invention, for example for use as subunit vaccines or in immunoassays.

The polynucleotides according to the invention have utility in the production by expression of the encoded proteins, which expression may take place *in vitro, in vivo* or *ex vivo*. The nucleotides may therefore be involved in recombinant protein synthesis, for example to increase yields, or indeed may find use as therapeutic agents in their own right, utilised in DNA vaccination techniques. Where the polynucleotides of the present invention are used in the production of the encoded proteins *in vitro* or *ex vivo*, cells, for example in cell culture, will be modified to include the polynucleotide to be expressed. Such cells include transient, or preferably stable mammalian cell lines. Particular examples of cells which may be modified by insertion of vectors encoding for a polypeptide according to the invention include mammalian HEK293T, CHO, HeLa, 293 and COS cells. Preferably the cell line selected will be one which is not only stable, but also allows for mature glycosylation and cell surface expression of a polypeptide. Expression may be achieved in transformed oocytes. A polypeptide may be expressed from a polynucleotide of the present invention, in cells of a transgenic non-human animal, preferably a mouse. A transgenic non-human animal expressing a polypeptide from a polynucleotide of the invention is included within the scope of the invention.

The invention further provides a method of vaccinating a mammalian subject which comprises administering thereto an effective amount of such a vaccine or vaccine composition. Most preferably, expression vectors for use in DNA vaccines, vaccine compositions and immunotherapeutics will be plasmid vectors.

DNA vaccines may be administered in the form of "naked DNA", for example in a liquid formulation administered using a syringe or high pressure jet, or DNA formulated with liposomes or an irritant transfection enhancer, or by particle mediated DNA delivery (PMDD). All of these delivery systems are well known in the art. The vector may be introduced to a mammal for example by means of a viral vector delivery system.

The compositions of the present invention can be delivered by a number of routes such as intramuscularly, subcutaneously, intraperitonally, intravenously or mucosally.

In a preferred embodiment, the composition is delivered intradermally. In particular, the composition is delivered by means of a gene gun particularly particle bombardment administration techniques which involve coating the vector on to a bead (eg gold) which are then administered under high pressure into the epidermis; such as, for example, as described in Haynes et al, J Biotechnology 44: 37-42 (1996).

In one illustrative example, gas-driven particle acceleration can be achieved with devices such as those manufactured by Powderject Pharmaceuticals PLC (Oxford, UK) and Powderject Vaccines Inc. (Madison, WI), some examples of which are described in U.S. Patent Nos. 5,846,796; 6,010,478; 5,865,796; 5,584,807; and EP Patent No. 0500 799. This approach offers a needle-free delivery approach wherein a dry powder formulation of microscopic particles, such as polynucleotide, are accelerated to high speed within a helium gas jet generated by a hand held device, propelling the particles into a target tissue of interest, typically the skin.

The particles are preferably gold beads of a 0.4 - 4.0 μm, more preferably 0.6 - 2.0 μm diameter and the DNA conjugate coated onto these and then encased in a cartridge or cassette for placing into the "gene gun".

In a related embodiment, other devices and methods that may be useful for gas-driven needle-less injection of compositions of the present invention include those provided by Bioject, Inc. (Portland, OR), some examples of which are described in U.S. Patent Nos. 4,790,824; 5,064,413; 5,312,335; 5,383,851; 5,399,163; 5,520,639 and 5,993,412.

The vectors which comprise the nucleotide sequences encoding antigenic peptides are administered in such amount as will be prophylactically or therapeutically effective. The quantity to be administered, is generally in the range of one picogram to 1 milligram, preferably 1 picogram to 10 micrograms for particle-mediated delivery, and 100 nanograms to 1 milligram, preferably 10 micrograms to 1 milligram, for other routes, of nucleotide per dose. The exact quantity may vary considerably depending on the weight of the patient being immunised and the route of administration,

It is possible for the immunogen component comprising the nucleotide sequence encoding the antigenic peptide, to be administered on a once off basis or to be administered repeatedly, for example, between 1 and 7 times, preferably between 1 and 4 times, at intervals between about 1 day and about 18 months. However, this treatment regime will be significantly varied depending upon the size the patient concerned, the amount of nucleotide sequence administered, the route of administration, and other factors which would be apparent to a skilled

veterinary or medical practitioner. The patient may receive one or more other anti HIV retroviral drugs as part of their overall treatment regime. Additionally the nucleic acid immunogen may be administered with an adjuvant.

The adjuvant component specified herein can similarly be administered via a variety of different administration routes, such as for example, via the oral, nasal, pulmonary, intramuscular, subcutaneous, intradermal or topical routes. Preferably, the adjuvant component is administered via the intradermal or topical routes. Most preferably by the topical route. This administration may take place between about 14 days prior to and about 14 days post administration of the nucleotide sequence, preferably between about 1day prior to and about 3 days post administration of the nucleotide sequence. The adjuvant component is, in an embodiment, administered substantially simultaneously with the administration of the nucleotide sequence. By "substantially simultaneous" what is meant is that administration of the adjuvant component is preferably at the same time as administration of the nucleotide sequence, or if not, at least within a few hours either side of nucleotide sequence administration. In the most preferred treatment protocol, the adjuvant component will be administered substantially simultaneously to administration of the nucleotide sequence. Obviously, this protocol can be varied as necessary, in accordance with the type of variables referred to above. It is preferred that the adjuvant is a 1H - imidazo [4,5c] quinoline - 4 - amine derivative such as imiquimod. Typically imiquimod will be presented as a topical cream formulation and will be administered according to the above protocol.

Once again, depending upon such variables, the dose of administration of the derivative will also vary, but may, for example, range between about 0.1mg per kg to about 100mg per kg, where "per kg" refers to the body weight of the mammal concerned. This administration of the 1H-imidazo[4,5-c]quinolin-4-amine derivative would preferably be repeated with each subsequent or booster administration of the nucleotide sequence. Most preferably, the administration dose will be between about 1mg per kg to about 50mg per kg. In the case of a "prim-boost" scheme as described herein, the imiquimod or other 1H-imidazo[4,5-c]quinolin-4-amine derivative may be administered with either the prime or the boost or with both the prime and the boost.

While it is possible for the adjuvant component to comprise only 1H-imidazo[4,5-c]quinolin-4-amine derivatives to be administered in the raw chemical state, it is preferable for administration to be in the form of a pharmaceutical formulation. That is, the adjuvant component will preferably comprise the 1H-imidazo[4,5-c]quinolin-4-amine combined with one or more pharmaceutically acceptable carriers, and optionally other therapeutic ingredients. The carrier(s) must be "acceptable" in the sense of being compatible with other ingredients within the formulation, and not deleterious to the recipient thereof. The nature of the formulations will naturally vary according to the intended administration route, and may be prepared by methods well known in the pharmaceutical art. All methods include the step of bringing into association a 1H-imidazo[4,5-c]quinolin-4-amine derivative with an appropriate carrier or carriers. In general, the formulations are prepared by uniformly and intimately bringing into association the derivative with liquid carriers or finely divided solid

carriers, or both, and then, if necessary, shaping the product into the desired formulation. Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets each containing a pre-determined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil emulsion. The active ingredient may also be presented as a bolus, electuary or paste.

A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent, lubricating, surface active or dispersing agent. Moulded tablets may be made by moulding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent.

The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient.

Formulations for injection via, for example, the intramuscular, intraperitoneal, or subcutaneous administration routes include aqueous and non-aqueous sterile injection solutions which may contain antioxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed

ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described. Formulations suitable for pulmonary administration via the buccal or nasal cavity are presented such that particles containing the active ingredient, desirably having a diameter in the range of 0.5 to 7 microns, are delivered into the bronchial tree of the recipient. Possibilities for such formulations are that they are in the form of finely comminuted powders which may conveniently be presented either in a piercable capsule, suitably of, for example, gelatine, for use in an inhalation device, or alternatively, as a self-propelling formulation comprising active ingredient, a suitable liquid propellant and optionally, other ingredients such as surfactant and/or a solid diluent. Self-propelling formulations may also be employed wherein the active ingredient is dispensed in the form of droplets of a solution or suspension. Such self-propelling formulations are analogous to those known in the art and may be prepared by established procedures. They are suitably provided with either a manually-operable or automatically functioning valve having the desired spray characteristics; advantageously the valve is of a metered type delivering a fixed volume, for example, 50 to 100 µL, upon each operation thereof.

In a further possibility, the adjuvant component may be in the form of a solution for use in an atomiser or nebuliser whereby an accelerated airstream or ultrasonic agitation is employed to produce a find droplet mist for inhalation.

Formulations suitable for intranasal administration generally include presentations similar to those described above for pulmonary administration, although it is preferred for such formulations to have a particle diameter in the range of about 10 to about 200 microns, to enable retention within the nasal cavity. This may be achieved by, as appropriate, use of a powder of a suitable particle size, or choice of an appropriate valve. Other suitable formulations include coarse powders having a particle diameter in the range of about 20 to about 500 microns, for administration by rapid inhalation through the nasal passage from a container held close up to the nose, and nasal drops comprising about 0.2 to 5% w/w of the active ingredient in aqueous or oily solutions. In one embodiment of the invention, it is possible for the vector which comprises the nucleotide sequence encoding the antigenic peptide to be administered within the same formulation as the 1H-imidazo[4,5-c]quinolin-4-amine derivative. Hence in this embodiment, the immunogenic and the adjuvant component are found within the same formulation.

In an embodiment the adjuvant component is prepared in a form suitable for gene-gun administration, and is administered via that route substantially simultaneous to administration of the nucleotide sequence. For preparation of formulations suitable for use in this manner, it may be necessary for the 1H-imidazo[4,5-c]quinolin-4-amine derivative to be lyophilised and adhered onto, for example, gold beads which are suited for gene-gun administration.

In an alternative embodiment, the adjuvant component may be administered as a dry powder, via high pressure gas propulsion.

Even if not formulated together, it may be appropriate for the adjuvant component to be administered at or about the same administration site as the nucleotide sequence.

Other details of pharmaceutical preparations can be found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pennysylvania (1985), the disclosure of which is included herein in its entirety, by way of reference.

Suitable techniques for introducing the naked polynucleotide or vector into a patient also include topical application with an appropriate vehicle. The nucleic acid may be administered topically to the skin, or to mucosal surfaces for example by intranasal, oral, intravaginal or intrarectal administration. The naked polynucleotide or vector may be present together with a pharmaceutically acceptable excipient, such as phosphate buffered saline (PBS). DNA uptake may be further facilitated by use of facilitating agents such as bupivacaine, either separately or included in the DNA formulation. Other methods of administering the nucleic acid directly to a recipient include ultrasound, electrical stimulation, electroporation and microseeding which is described in US-5,697,901.

Uptake of nucleic acid constructs may be enhanced by several known transfection techniques, for example those including the use of transfection agents. Examples of these agents includes cationic agents, for example, calcium phosphate and DEAE-Dextran and lipofectants, for

example, lipofectam and transfectam. The dosage of the nucleic acid to be administered can be altered.

A nucleic acid sequence of the present invention may also be administered by means of specialised delivery vectors useful in gene therapy. Gene therapy approaches are discussed for example by Verme *et al*, Nature 1997, **389**:239-242. Both viral and non-viral vector systems can be used. Viral based systems include retroviral, lentiviral, adenoviral, adeno-associated viral, herpes viral, Canarypox and vaccinia-viral based systems. Non-viral based systems include direct administration of nucleic acids, microsphere encapsulation technology (poly(lactide-co-glycolide) and, liposome-based systems. Viral and non-viral delivery systems may be combined where it is desirable to provide booster injections after an initial vaccination, for example an initial "prime" DNA vaccination using a non-viral vector such as a plasmid followed by one or more "boost" vaccinations using a viral vector or non-viral based system. Similarly the invention contemplates prime boot systems with the polynucleotide of the invention, followed by boosting with protein in adjuvant or vice versa.

A nucleic acid sequence of the present invention may also be administered by means of transformed cells. Such cells include cells harvested from a subject. The naked polynucleotide or vector of the present invention can be introduced into such cells *in vitro* and the transformed cells can later be returned to the subject. The polynucleotide of the invention may integrate into nucleic acid already present in a cell by homologous recombination events. A transformed cell may, if desired, be grown up *in vitro* and one or more of the resultant cells may be used in the present invention.

Cells can be provided at an appropriate site in a patient by known surgical or microsurgical techniques (e.g. grafting, micro-injection, etc.)

The pharmaceutical compositions of the present invention may include adjuvant compounds, as detailed above, or other substances which may serve to increase the immune response induced by the protein which is encoded by the DNA. These may be encoded by the DNA, either separately from or as a fusion with the antigen, or may be included as non-DNA elements of the formulation. Examples of adjuvant-type substances which may be included in the formulations of the present invention include ubiquitin, lysosomal associated membrane protein (LAMP), hepatitis B virus core antigen, FLT3-ligand (a cytokine important in the generation of professional antigen presenting cells, particularly dentritic cells) and other cytokines such as IFN-γ and GMCSF. Other preferred adjuvants include Imiquimod and Resimquimod and Tucarasol. Imiquimod being particularly preferred.

The present invention in a preferred embodiments of the invention provides the use of a nucleic acid molecule as herein described for the treatment or prophylaxis of HIV infection. The nucleic acid molecule is preferably administered with Imiquimod. The Imiquimod is preferably administered topically, whereas the nucleic acid molecule is preferably administered by means of the particle mediated delivery.

Accordingly the present invention provides a method of treating a subject suffering from or susceptible to HIV infection, comprising administering a nucleic acid molecule as herein described and Imiquimod.

The present invention will now be described by reference to the following examples:

EXAMPLES

Example 1: Optimisation of p55 gag (p17, p24, p13) to resemble codon usage of highly expressed human genes.

Gene of interest

A synthetic gene coding for the p55gag antigen of the HIV-1 clade B strain HXB2 (GenBank entry K03455), optimised for expression in mammalian cells was assembled from overlapping oligonucleotides by PCR.

Optimisation involved changing the codon usage pattern of the viral gene to give a codon frequency closer to that found in highly expressed human genes. Codons were assigned using a statistical Visual Basic program called Syngene (an updated version of Calcgene, written by R.S. Hale and G. Thompson, Protein Expression and Purification Vol. 12 pp 185-188, 1998)

Cloning:

The 1528bp gag PCR product was gel purified, cut with restriction endonucleases NotI and Bam HI and ligated into NotI/BamHI cut vector WRG7077. This places the gene between the CMV promoter/intron A and the Bovine growth hormone polyadenylation signal.

Clones were sequenced and checked for errors. No single clone was 100% correct. Regions of correct sequence from two clones were therefore combined by overlapping PCR

using appropriate combinations of the optimisation oligo set to give a full length codon optimised gag gene. This final clone was subsequently found to contain a single nucleotide deletion which resulted in a frame shift and premature termination of translation. The deletion was repaired by cutting out the region of the gene containing the incorrect sequence and cloning in the correct sequence from the equivalent region of another clone. This gave the final codon optimised p55 gag clone: Gagoptrpr2. (See figure 2)

Example 2: Production of a p17/p24 truncated Nef fusion gene

Gene of interest

The p17 and p24 portions of the p55gag gene derived from the HIV-1 clade B strain HXB2 was PCR amplified from the plasmid pHXB?Pr (B.Maschera, E Furfine and E.D. Blair 1995 J.Virol **69** 5431-5436). pHXB?Pr. 426bp from the 3′ end of the HXB2 nef gene were amplified from the same plasmid. Since the HXB2 nef gene contains a premature termination codon two overlapping PCRs were used to repair the codon (TGA [stop] to TGG [Trp])

The p17/p24linker and trNEFlinker PCR products were joined to form the p17p24trNEF fusion gene (figure 3) in a PCR reaction (**antisense**)

The 1542bp product was gel purified, cut with restriction endonucleases NotI and BamHI and cloned into the NotI BamHI sites of vector WRG7077. This places the gene between the CMV promoter/intron A and the Bovine growth hormone polyadenylation signal.

Example 3: Production of an Gag p17/24opt/trNef1 ('Gagopt/Nef') fusion gene.

Gene of interest

The p17/p24 portion of the codon optimised p55gag gene derived from the HIV-1 clade B strain HXB2 was PCR amplified from the plasmid pGagOPTrpr2. The truncated HXB2 Nef gene with the premature termination codon repaired (TGA [stop] to TGG [Trp]) was amplified by PCR from the plasmid 7077trNef20. The two PCR products were designed to have overlapping ends so that the two genes could be joined in a second PCR.

The 1544bp product was gel purified, cut with restriction endonucleases NotI and BamHI and cloned (see figures) into the NotI BamHI sites of vector WRG7077. This places the gene between the CMV promoter/intron A and the Bovine growth hormone polyadenylation signal.

Example 4: Plasmid: p7077-RT3 Clone #A

Gene of interest:

A synthetic gene coding for the RT portion of the pol gene of HIV-1 clade B strain HXB2, optimised for expression in mammalian cells assembled from overlapping oligonucleotides by PCR. The sequence cloned is equivalent to positions 2550-4222 of the HXB2 reference sequence (GenBank entry K03455). To ensure expression the cloned sequence has two additional codons at the 5′ end not present in the original gene – AUG GGC (Met Gly).

Optimisation involved changing the codon usage pattern of the viral gene to give a codon frequency closer to that found in highly expressed human genes, but excluding rarely used codons. Codons were assigned using a statistical Visual Basic program called Syngene (an updated version of Calcgene, written by R.S. Hale and G. Thompson, Protein Expression and Purification Vol. 12 pp 185-188, 1998)

The final clone was constructed from two intermediate clones, # 16 and #21.

Cloning:

The 1.7kb PCR products were gel purified, cut with NotI and BamHI and PCR cleaned, before being ligated with NotI / BamHI cut pWRG7077. This places the gene between the CMV promoter and bovine growth hormone polyadenylation signal. Clones were sequenced. No clone was 100% correct, but clone #16 was corrected by replacing the 403bp KpnI-BamHI fragment containing 3 errors with a correct Kpn1-BamHI fragment from clone#21. The final clone was verified by sequencing. (see figure 5)

Example 5: Optimised RT

Gene of interest

The synthetic gene coding for the RT portion of the pol gene of HIV-1 clade B strain HXB2, optimised for expression in mammalian cells was excised from plasmid p7077-RT3 as a 1697bp NotI / BamHI fragment, gel purified, and cloned into the NotI & BamHI sites of p7313-ie (derived from pspC31) to place the gene

downstream of an Iowa length HCMV promoter + exon1, and upstream of a rabbit globin poly-adenylation signal. (R7004 p27) (figure 6)

Example 6

Plasmid: 7077trNef20

Gene of interest

The insert comprises part of the Nef gene from the HIV-1 clade B strain HXB2. 195bp are deleted from the 5′ end of the gene removing the codons for the first 65 amino acids of Nef. In addition the premature termination codon in the published HXB2 nef sequence has been repaired (TAG to TGG [Trp]) as has been described for plasmid p17/24trNEF1. The truncated nef sequence was PCR amplified from the plasmid p17/24trNef1. The sequence cloned is equivalent to positions 8992-9417 of the HXB2 reference sequence (GenBank entry K03455). To ensure expression the cloned sequence has an additional codon at the 5′ end not present in the original gene - AUG (Met).

Primers:

StrNef (sense) ATAAGAATGCGGCCGCCATGGTGGGTTTTCCAGTCACACCTT [SEQ ID NO: 1]

AStrNef (antisense)

CGCGGATCCTCAGCAGTTCTTGAAGTACTCC [SEQ ID NO: 2]

PCR: 94° C 2min, then 25 cycles: 94° C 30sec, 50° C 30sec, 72° C 2min, ending 72° C 5min

Cloning:

The 455bp RT PCR product was gel purified, cut with restriction endonucleases NotI and Bam HI and ligated into NotI/BamHI cut vector WRG7077. This places the gene

between the CMV promoter/intron A and the Bovine growth hormone polyadenylation signal.

Example 7

Plasmid: 7077RT 8

Gene of interest

The RT portion of the pol gene was derived from the HIV-1 clade B strain HXB2. It was PCR amplified from the plasmid p7077Pol14.

The sequence cloned is equivalent to positions 2550-4234 of the HXB2 reference sequence (GenBank entry K03455). To ensure expression the cloned sequence has two additional codons at the 5′ end not present in the original gene – AUG GGC (Met Gly).

Primers:

SRT (sense) ATAAGAATGCGGCCGCCATGGGCCCCATTAGCCCTATTGAGACT [SEQ ID NO: 3]

ASRT (antisense)

CGCGGATCCTTAATCTAAAAATAGTACTTTCCTGATT [SEQ ID NO: 4]

PCR: 94° C 2min, then 25 cycles: 94° C 30sec, 50° C 30sec, 72° C 4min, ending 72° C 5min

Cloning:

The 1720bp RT PCR product was gel purified, cut with restriction endonucleases NotI and Bam HI and ligated into NotI/BamHI cut vector WRG7077. This places the gene between the CMV promoter/intron A and the Bovine growth hormone polyadenylation signal.

Example 8

p17/24opt/RT/trNef13 ('Gagopt/RT/Nef')

This construct contains a PCR that causes an R to H amino acid change.

Gene of interest:

The p17/p24 portion of the codon optimised p55gag gene derived from the HIV-1 clade B strain HXB2 was PCR amplified from the plasmid pGagOPTrpr2. The RT coding sequence was PCR amplified from the plasmid 7077RT 8. The truncated HXB2 Nef gene with the premature termination codon repaired (TGA [stop] to TGG [Trp]) was amplified by PCR from the plasmid 7077trNef20. The three PCR products were designed to have overlapping ends so that the three genes could be joined in a second PCR.

Primers:

(P17/24)

Sp17p24opt (sense)
ATAAGAATGCGGCCGCCATGGGTGCCCGAGCTTCGGT [SEQ ID NO: 5]
ASp17p24optRTlinker (antisense)
TGGGGCCCATCAACACTCTGGCTTTGTGTC [SEQ ID NO: 6]

PCR: 94° C 1min, then 20 cycles: 94° C 30sec, 50° C 30sec, 72° C 2min, ending 72° C 4min

The 1114bp p17/24opt product was gel purified.

(RT)

Sp17p24optRTlinker (sense)
CAGAGTGTTGATGGGCCCCATTAGCCCTAT [SEQ ID NO: 7]
ASRTtrNeflinker (antisense)

AACCCACCATATCTAAAAATAGTACTTTCC [SEQ ID NO: 8]

PCR: as above

The 1711bp RT PCR product was gel purified

(5' truncated nef)

SRTtrNef linker (sense)
CTATTTTTAGATATGGTGGGTTTTCCAGTCAC [SEQ ID NO: 9]
AStrNef (antisense)
CGCGGATCCTCAGCAGTTCTTGAAGTACTCC [SEQ ID NO: 10]

PCR as above.
The 448bp product was gel purified.

The three PCR products were then stitched together in a second PCR with primers Sp17/24opt and AstrNef.

PCR: 94° C 1min, then 30 cycles: 94° C 30sec, 50° C 30sec, 72° C 4min, ending 72° C 4min

The 3253bp product was gel purified, cut with restriction endonucleases NotI and BamHI and cloned into the NotI BamHI sites of vector WRG7077. This places the gene between the CMV promoter/intron A and the Bovine growth hormone polyadenylation signal.

Example 9

Plasmid: pGRN#16 (p17/p24opt corr /RT/trNef.)

Gene of interest:
The polyprotein generated by p17/24opt/RT/trNef13 ('Gagopt/RT/Nef') was observed to express a truncated product of ~30kDa due to a cluster of unfavourable codons

within p24 around aminoacid 270. These were replaced with optimal codons by PCR stitching mutagenesis. p17/24opt/RT/trNef13 was used as a template to amplify the portion of Gag 5' to the mutation with primers Sp17/p24opt and GTR-A, and the portion of Gag 3' to the mutation with primers GTR-S and Asp17/p24optRTlinker. The overlap of the products contained the codon changes, and the gel purified products were stitched together using the Sp17/p24opt and Asp17/p24optRTlinker primers. The product was cut with NotI and AgeI and inserted into similarly cut p17/24opt/RT/trNef13, to generate pGRN. Clone #16 was verified and progressed.

Primers:

5' PCR:

Sp17p24opt (sense)
ATAAGAATGCGGCCGCCATGGGTGCCCGAGCTTCGGT [SEQ ID NO: 11]

GTR-A (Antisense)
GCGCACGATCTTGTTCAGGCCCAGGATGATCCACCGTTTATAGATTTCTCC [SEQ ID NO: 12]

3' PCR
Sense: GTR-S(Sense)
ATCCTGGGCCTGAACAAGATCGTGCGCATGTACTCTCCGACATCCATCC [SEQ ID NO: 13]

ASp17p24optRTlinker (antisense)
TGGGGCCCATCAACACTCTGGCTTTGTGTC [SEQ ID NO: 14]

PCR conditions for individual products and stitch, using PWO DNA polymerase (Roche):

95°C 1min, then 20 cycles 95°C 30s, 55°C 30s, 72°C 180s, ending 72°C 120s and 4°C hold.

The 1114bp product was gel purified and cut with NotI and AgeI to release a 6647bp fragment which was gel purified and ligated into NotI / AgeI cut gel purified p17/24opt/RT/trNef13 to generate pGRN#16.

Example 10:

Plasmid: p73i-GRN2 Clone #19
(p17/p24(opt)/RT(opt)trNef) - repaired

Gene of interest:
The p17/p24 portion of the codon optimised gag, codon optimised RT and truncated Nef gene from the HIV-1 clade B strain HXB2 downstream of an Iowa length HCMV promoter + exon1, and upstream of a rabbit β-globin poly-adenylation signal.

Plasmids containing the trNef gene derived from plasmid p17/24trNef1 contain a PCR error that gives an R to H amino acid change 19 amino acids from the end of nef. This was corrected by PCR mutagenesis, the corrected nef PCR stitched to codon optimised RT from p7077-RT3, and the stitched fragment cut with ApaI and BamHI, and cloned into ApaI/BamHI cut p73i-GRN.

Primers:
PCR coRT from p7077-RT3 using primers:
(Polymerase = PWO (Roche) throughout.

Sense: U1
GAATTCGCGGCCGCGATGGGCCCCATCAGTCCCATCGAGACCGTGCCGGTGAAGCTG AAACCCGGGAT [SEQ ID NO: 15]

AScoRT-Nef
GGTGTGACTGGAAAACCCACCATCAGCACCTTTCTAATCCCCGC [SEQ ID NO: 16]

Cycle: 95°C(30s) then 20 cycles 95°C(30s), 55°C(30s), 72°C(180s), then 72°C(120s) and hold at 4°C
The 1.7kb PCR product was gel purified.

PCR 5′ Nef from p17/24trNef1 using primers:

Sense: S-Nef
ATGGTGGGTTTTCCAGTCACACC [SEQ ID NO: 17]
Antisense: ASNef-G:
GATGAAATGCTAGGCGGCTGTCAAACCTC [SEQ ID NO: 18]
Cycle: 95°C(30s) then 15 cycles 95°C(30s), 55°C(30s), 72°C(60s), then 72°C(120s) and hold at 4°C

PCR 3′ Nef from p17/24trNef1 using primers:

Sense: SNEF-G
GAGGTTTGACAGCCGCCTAGCATTTCATC [SEQ ID NO: 19]
Antisense:
AStrNef (antisense)
CGCGGATCCTCAGCAGTTCTTGAAGTACTCC [SEQ ID NO: 20]
Cycle: 95°C(30s) then 15 cycles 95°C(30s), 55°C(30s), 72°C(60s), then 72°C(120s) and hold at 4°C

The PCR products were gel purified. Initially the two Nef products were stitched using the 5′ (S-Nef) and 3′ (AstrNef) primers.
Cycle: 95°C(30s) then 15 cycles 95°C(30s), 55°C(30s), 72°C(60s), then 72°C(180s) and hold at 4°C .
The PCR product was PCR cleaned, and stitched to the RT product using the U1 and AstrNef primers:

Cycle: 95°C(30s) then 20 cycles 95°C(30s), 55°C(30s), 72°C(180s), then 72°C(180s) and hold at 4°C

The 2.1kb product was gel purified, and cut with ApaI and BamHI. The plasmid p73I-GRN was also cut with Apa1 and BamHI gel purified and ligated with the ApaI-Bam RT3trNef to regenerate the p17/p24(opt)/RT(opt)trNef gene.

Example 11

p73i-GN2 Clone #2 (p17/p24opt/trNef) - repaired

Gene of interest:

The p17/p24 portion of the codon optimised gag and truncated Nef genes from the HIV-1 clade B strain HXB2 downstream of an Iowa length HCMV promoter + exon1, and upstream of a rabbit β-globin poly-adenylation signal.

Plasmids containing the trNef gene derived from plasmid p17/24trNef1 contain a PCR error that gives an R to H amino acid change 19 amino acids from the end of Nef. This was corrected by PCR mutagenesis and the corrected fragment cut with BglII and BamHI, and cloned into BglII/BamHI cut p73I-GN. (Figure 12) regenerate the corrected p17/p24opt/trNef fusion gene downstream of the Iowa length HCMV promoter + exon1, and upstream of the rabbit β-globin polyadenylation signal.

PCR 5' Nef from p17/24trNef1 using primers:

Polymerase = PWO (Roche) throughout.

Sense: S-Nef

ATGGTGGGTTTTCCAGTCACACC [SEQ ID NO: 21]

Antisense: ASNef-G:

GATGAAATGCTAGGCGGCTGTCAAACCTC [SEQ ID NO: 22]

Cycle: 95°C(30s) then 15 cycles 95°C(30s), 55°C(30s), 72°C(60s), then 72°C(120s) and hold at 4°C

PCR 3' Nef from p17/24trNef1 using primers:

Sense: SNEF-G

GAGGTTTGACAGCCGCCTAGCATTTCATC [SEQ ID NO: 23]

Antisense: AStrNef

CGCGGATCCTCAGCAGTTCTTGAAGTACTCC [SEQ ID NO: 24]

Cycle: 95°C(30s) then 15 cycles 95°C(30s), 55°C(30s), 72°C(60s), then 72°C(120s) and hold at 4°C

The PCR products were gel purified, and stitched using the 5' (S-Nef) and 3' (AstrNef) primers.

Cycle: 95°C(30s) then 15 cycles 95°C(30s), 55°C(30s), 72°C(60s), then 72°C(180s) and hold at 4°C .

The PCR product was PCR cleaned, cut with BglII / BamHI , and the 367bp fragment gel purified and cloned into BglII/BamHI cut gel purified p73i-GN.

Example 12

Plasmid: p73I-RT w229k (Inactivated RT)

Gene of Interest:

Generation of an inactivated RT gene downstream of an Iowa length HCMV promoter + exon 1, and upstream of a rabbit β-globin poly-adenylation signal.

Due to concerns over the use of an active HIV RT species in a therapeutic vaccine inactivation of the gene was desirable. This was achieved by PCR mutagenesis of the RT (derived from P73I-GRN2) amino acid position 229 from Trp to Lys (R7271 p1-28).

Primers:

PCR 5′ RT + mutation using primers:
(polymerase = PWO (Roche) throughout)

Sense : RT3-u:1
GAATTCGCGGCCGCGATGGGCCCCATCAGTCCCATCGAGACCGTGCCGGTGAAGCTG
AAACCCGGGAT [SEQ ID NO: 25]

Antisense: AScoRT-Trp229Lys
GGAGCTCGTAGCCCATCTTCAGGAATGGCGGCTCCTTCT [SEQ ID NO: 26]

Cycle:
1 x [94°C (30s)]
15 x [94°C (30s)/55°C (30s)/72°C (60s)]
1 x [72°C (180s)]
PCR gel purify

PCR 3′ RT + mutation using primers:

Antiense: RT3- 1:1
GAATTCGGATCCTTACAGCACCTTTCTAATCCCCGCACTCACCAGCTTGTCGACCTG
CTCGTTGCCGC [SEQ ID NO: 27]

Sense: ScoRT-Trp229Lys
CCTGAAGATGGGCTACGAGCTCCATG [SEQ ID NO: 28]

Cycle:
1 x [94°C (30s)]
15 x [94°C (30s)/55°C (30s)/72°C (60s)]
1 x [72°C (180s)]
PCR gel purify

The PCR products were gel purified and the 5′ and 3′ ends of RT were stitched using the 5′ (RT3-U1) and 3′ (RT3-L1) primers.

Cycle:
1 x [94°C (30s)]
15 x [94°C (30s)/55°C (30s)/72°C (120s)]
1 x [72°C (180s)]

The PCR product was gel purified, and cloned into p7313ie, utilising NotI and BamHI restriction sites, to generate p73I-RT w229k. (See figure 13)

Example 13:

Plasmid: p73i-Tgrn (#3)

Gene of interest:

The p17/p24 portion of the codon optimised gag, codon optimised RT and truncated Nef gene from the HIV-1 clade B strain HXB2 downstream of an Iowa length HCMV promoter + exon1, and upstream of a rabbit β-globin poly-adenylation signal.

Triple fusion constructs which contain an active form of RT, may not be acceptable to regulatory authorities for human use thus inactivation of RT was achieved by Insertion of a NheI and ApaI cut fragment from p73i-RT w229k, into NheI/ApaI cut p73i-GRN2#19 (Figure 14). This results in a W → K change at position 229 in RT.

Example 14

p73I-Tnrg (#16)

Gene of interest:

The truncated Nef , inactivated codon optimised RT and p17/p24 portion of the codon optimised gag gene from the

HIV-1 clade B strain HXB2 downstream of an Iowa length HCMV promoter + exon1, and upstream of a rabbit β-globin poly-adenylation signal.

The order of the genes in the polyprotein encoded by p73i-Tgrn were rearranged by PCR and PCR stitching to generate p73I-Tnrg (Figure 15). Each gene was PCR amplified and gel purified prior to PCR stitching of the genes to form a single polyprotein. The product was gel purified, NotI/BamHI digested and ligated into NotI/BamHI cut p7313ie.

Primers:

trNef PCR

S-Nef (Not I)
CATTAGAGCGGCCGCGATGGTGGGTTTTCCAC [SEQ ID NO: 29]

AS-Nef-coRT linker
GATGGGACTGATGGGGCCCATGCAGTTCTTGAACTACTCCGG [SEQ ID NO: 30]

RTw229k PCR

S-coRT
ATGGGCCCCATCAGTCCCATCGAG [SEQ ID NO: 31]

AS-coRT-p17p24 linker
CAGTACCGAAGCTCGGGCACCCATCAGCACCTTTCTAATCCCCGC [SEQ ID NO: 32]

p17p24opt PCR

S-p17p24opt
ATGGGTGCCCGAGCTTCGGTACTG [SEQ ID NO: 33]

AS-p17p24opt (BamHI)
GATGGGGGATCCTCACAACACTCTGGCTTTGTGTCC [SEQ ID NO: 34]

PCR conditions for individual products and stitching using VENT DNA polymerase (NEB):

1 x [94°C (30s)]
25 x [94°C (30s)/55°C (30s)/72°C (120s [p17p24 or RT] or 60s [trNef])]
1 x [72°C (240s)]

The PCR products were gel purified and used in a PCR stitching utilising the primers S-trNef (*Not*I) and AS-p17p24opt (*Bam*HI):

1 x [94°C (30s)]
25 x [94°C (30s)/55°C (30s)/72°C (210s)]
1 x [72°C (240s)]

The 3000bp product was gel purified and cut with NotI and BamHI which was PCR cleaned and ligated into NotI/BamHI digested gel purified p7313ie to generate p73i-Tnrg.

Example 15:

1. Plasmid: P73i-Tngr (#3)

Gene of Interest:
The truncated Nef , p17/p24 portion of the codon optimised gag and inactivated codon optimised RT gene from the HIV-1 clade B strain HXB2 downstream of an Iowa length HCMV promoter + exon1, and upstream of a rabbit β-globin poly-adenylation signal.

The order of the genes in the polyprotein encoded by p73i-Tgrn were rearranged by PCR to generate p73I-Tngr (Figure 16). Codon optimised p17/p24 and RT were generated as a single product, and PCR stitched to amplified trNef. The product was gel purified, NotI/BamHI digested and ligated into NotI/BamHI cut p7313ie.

Primers:

P17/p24 - RT 3'PCR:

Sp17p24opt (sense)
ATGGGTGCCCGAGCTTCGGTACTG [SEQ ID NO: 35]

RT3 1:1 (antisense)
GAATTCGGATCCTTACAGCACCTTTCTAATCCCCGCACTCACCAGCTTGTCGACCTG CTCGTTGCCGC [SEQ ID NO: 36]

TrNef 5'PCR

S-Nef (NotI)
CATTAGAGCGGCCGCGATGGTGGGTTTTCCAC [SEQ ID NO: 37]

AS-Nef-p17p24
CAGTACCGAAGCTCGGGCACCCATGCAGTTCTTGAACTACTCCGG [SEQ ID NO: 38]

PCR conditions for individual products and stitching using VENT DNA polymerase (NEB):

1 x [94°C (30s)]
25 x [94°C (30s)/55°C (30s)/72°C (180s [p17p24+RT] or 60s [trNef] or 210s [stitching])]
1 x [72°C (240s)]

The 3000bp product was gel purified and cut with NotI and BamHI which was PCR cleaned and ligated into NotI/BamHI digested gel purified p7313ie to generate p73i-Tngr.

Example 16:

Plasmid: p73I-Trgn (#6)

Gene of interest:

The inactivated codon optimised RT, p17/p24 portion of the codon optimised gag and truncated Nef gene from the HIV-1 clade B strain HXB2 downstream of an Iowa length HCMV promoter + exon1, and upstream of a rabbit β-globin poly-adenylation signal.

The order of the genes within the construct was achieved by PCR amplification of p17p24-trNef and RTw229k from the plasmids p73I-GN2 and p73I-RTw229k respectively. PCR stitching was performed and the product gel purified and NotI/BamHI cut prior to ligation with NotI/BamHI digested p7313ie. Sequencing revealed that p17p24 was not fully optimised a 700bp fragment was then AgeI/MunI cut from the coding region and replaced with MunI/Age fragment from p73i-Tgrn#3 containing the correct coding sequence. (See figure 17).

Primers:

p17p24-trNef PCR

S-p17p24opt

ATGGGTGCCCGAGCTTCGGTACTG [SEQ ID NO: 39]

AstrNef (BamHI)

RTw229k

RT3-U:1
GAATTCGCGGCCGCGATGGGCCCCATCAGTCCCATCGAGACCGTGCCGGTGAAGCTG
AAACCCGGGAT [SEQ ID NO: 40]

AS-coRT-p17p24opt linker
CAGTACCGAAGCTCGGGCACCCATCAGCACCTTTCTAATCCCCGC [SEQ ID NO: 41]

PCR conditions for individual products and stitching using VENT DNA polymerase (NEB):

1 x [94°C (30s)]
25 x [94°C (30s)/55°C (30s)/72°C (120s (PCR) or 180s (stitching)
1 x [72°C (240s)]

The 3000bp product from the PCR stitch was gel purified and cut with NotI and BamHI which was PCR cleaned and ligated into NotI/BamHI digested gel purified p7313ie to generate p73i-Tngr. Sequence analysis showed that p17p24 sequence obtained from p73I-GN2 was not fully codon optimised and that this had been carried over into the new plasmid. This was rectified by cutting a 700bp fragment from p73i-Tngr cut with MunI and AgeI, and replacing it by ligation with a 700bp MunI/AgeI digested product from p73i-Tgrn to generate the construct p73I-Tngr#6.

Example 17:

Plasmid: p73i-Trng (#11)

Gene of Interest:

The inactivated codon optimised RT, truncated Nef and p17/p24 portion of the codon optimised gag gene from the HIV-1 clade B strain HXB2 downstream of an Iowa length HCMV promoter + exon1, and upstream of a rabbit β-globin poly-adenylation signal.

The order of the genes within the construct was achieved by PCR amplification of the RT-trNef and p17p24 genes from p73i-Tgrn. PCR stitching of the two DNA fragments was performed and the 3kb product gel purified and NotI/BamHI cut prior to ligation with NotI/BamHI digested p7313ie, and yielded p73I Trng (#11).

Primers:

RTw229k-trNef

RT3-u:1
GAATTCGCGGCCGCGATGGGCCCCATCAGTCCCATCGAGACCGTGCCGGTGAAGCTG AAACCCGGGAT [SEQ ID NO: 42]

AS-Nef-p17p24opt linker
CAGTACCGAAGCTCGGGCACCCATGCAGTTCTTGAACTACTCCGG [SEQ ID NO: 43]

P17p24

S-p17p24opt
ATGGGTGCCCGAGCTTCGGTACTG [SEQ ID NO: 44]

AS-p17p24opt(BamHI)
GATGGGGGATCCTCACAACACTCTGGCTTTGTGTCC [SEQ ID NO: 45]

PCR conditions for individual products and stitching using VENT DNA polymerase (NEB):

1 x [94°C (30s)]
25 x [94°C (30s)/55°C (30s)/72°C (120s (PCR of genes) or 180s (stitching)
1 x [72°C (240s)]

The 3000bp product from the PCR stitch was gel purified and cut with NotI and BamHI which was PCR cleaned and ligated into NotI/BamHI digested gel purified p7313ie to generate p73i-Tngr.

Example 18:

p73i-Tgnr (#f1)

Gene of interest:
The p17/p24 portion of the codon optimised gag, truncated Nef and codon optimised inactivated RT gene from the HIV-1 clade B strain HXB2 downstream of an Iowa length HCMV promoter + exon1, and upstream of a rabbit β-globin poly-adenylation signal.

The order of the genes within the construct was achieved by PCR amplification of p17p24-trNef and RTw229k from the plasmids p73I-GN2 and p73I-RTw229k respectively. PCR stitching was performed and the product gel purified and NotI/BamHI cut prior to ligation with NotI/BamHI digested p7313ie. Two sequence errors were spotted in the sequence (p17p24 and RT) which were subsequently repaired by replacement with correct portions of the genes utilising restriction sites within the polyprotein. (See figure 19).

Primers:

p17p24-trNef PCR

S-p17p24opt
ATGGGTGCCCGAGCTTCGGTACTG [SEQ ID NO: 46]

AS-Nef-coRTlinker
GATGGGACTGATGGGGCCCATGCAGTTCTTGAACTACTCCGG [SEQ ID NO: 47]

RTw229k

S-coRT
ATGGGCCCCATCAGTCCCATCGAG [SEQ ID NO: 48]

RT3-l:1
GAATTCGGATCCTTACAGCACCTTTCTAATCCCCGCACTCACCAGCTTGTCGACCTG CTCGTTGCCGC [SEQ ID NO: 49]

PCR conditions for individual products and stitching using VENT DNA polymerase (NEB):

1 x [94°C (30s)]
25 x [94°C (30s)/55°C (30s)/72°C (120s (PCR) or 180s (stitching)
1 x [72°C (240s)]

The 3000bp product was gel purified and cut with NotI and BamHI which was PCR cleaned and ligated into NotI/BamHI digested gel purified p7313ie to generate p73i-Tngr. Sequencing revealed that p17p24 was not fully optimised a 700bp fragment was subsequently AgeI/MunI cut from the coding region and replaced with MunI/Age fragment from p73i-Tgrn#3 containing the correct coding sequence. The polyprotein also contained a single point mutation (G2609A) resulting in an amino acid substitution of Thr

to Ala in the RT portion of the polyprotein. The mutation was corrected by ApaI/BamHI digestion of the construct and PCR clean up to remove the mutated sequence, which was replaced by ligation with an ApaI/BamHI digested portion of RT from p73i-Tgnr.

Example 19:
Preparation of plasmid-coated 'gold slurry' for 'gene gun' DNA cartridges

Plasmid DNA (approximately 1µg/µl), eg. 100 ug, and 2µm gold particles, eg. 50 mg, (PowderJect), were suspended in 0.05M spermidine, eg. 100 ul, (Sigma). The DNA was precipitated on to the gold particles by addition of 1M $CaCl_2$, eg. 100ul (American Pharmaceutical Partners, Inc., USA). The DNA/gold complex was incubated for 10 minutes at room temperature, washed 3 times in absolute ethanol, eg. 3 x 1 ml, (previously dried on molecular sieve 3A (BDH)). Samples were resuspended in absolute ethanol containing 0.05mg/ml of polyvinylpyrrolidone (PVP, Sigma), and split into three equal aliquots in 1.5 ml microfuge tubes, (Eppendorf). The aliquots were for analysis of (a) 'gold slurry', (b) eluate- plasmid eluted from (a) and (c) for preparation of gold/ plasmid coated Tefzel cartridges for the 'gene gun', (see Example 3 below). For preparation of samples (a) and (b), the tubes containing plasmid DNA / 'gold slurry' in ethanol / PVP were spun for 2 minutes at top speed in an Eppendorf 5418 microfuge, the supernatant was removed and the 'gold slurry' dried for 10 minutes at room temperature. Sample (a) was resuspended to 0.5 - 1.0 ug / ul of plasmid DNA in TE pH 8.0, assuming approx. 50 % coating. For elution, sample (b) was resuspended to 0.5 - 1.0 ug / ul of plasmid DNA in TE pH 8.0 and incubated at 37°C for 30 minutes, shaking vigorously, and then spun for 2 minutes

at top speed in an Eppendorf 5418 microfuge and the supernatant, eluate, was removed and stored at -20°C. The exact DNA concentration eluted was determined by spectrophotometric quantitation using a Genequant II (Pharmacia Biotech).

Example 20:
Preparations of Cartridges for DNA immunisation

Preparation of cartridges for the Accell gene transfer device was as previously described (Eisenbraun et al DNA and Cell Biology, 1993 Vol 12 No 9 pp 791-797; Pertner et al). Briefly, plasmid DNA was coated onto 2 μm gold particles (DeGussa Corp., South Plainfield, N.J., USA) and loaded into Tefzel tubing, which was subsequently cut into 1.27 cm lengths to serve as cartridges and stored desiccated at 4°C until use. In a typical vaccination, each cartridge contained 0.5 mg gold coated with a total of 0.5 μg DNA/cartridge.

Example 21:
Immune Response to HIV antigens following DNA vaccination utilising the gene gun.

Mice (n=3/group) were vaccinated with antigens encoded by nucleic acid and located in two vectors. P7077 utilises the HCMV IE promoter including Intron A and exon 1 (fcmv promoter). P73I delivers the same antigen, but contains the HCMV IE promoter (icmv promoter) that is devoid of Intron A, but includes exon 1.

Plasmid was delivered to the shaved target site of abdominal skin of F1 (C3H x Balb/c) mice. Mice were given a primary immunisation of 2 x 0.5 μg DNA on day 0, boosted with 2 x 0.5 μg DNA on day 35 and cellular

response were detected on day 40 using IFN – gamma Elispot.

- P73I - empty vector
- P7077 - empty vector
- P7077 GRN - (f CMV promoter) Gag, RT, Nef
- P73I GRN - (i CMV promoter) Gag, RT, Nef
- P73I GR3N - (CMV promoter) Optimised Gag, Optimised RT, Nef
- P7077 GN - (f CMV promoter)Gag, Nef
- P73I GN - (i CMV promoter) Gag, Nef

Cytotoxic T Cell Responses

The cytotoxic T cell response was assessed by CD8+ T cell-restricted IFN-γ ELISPOT assay of splenocytes collected 5 days later. Mice were killed by cervical dislocation and spleens were collected into ice-cold PBS. Splenocytes were teased out into phosphate buffered saline (PBS) followed by lysis of red blood cells (1 minute in buffer consisting of 155mM NH_4Cl, 10 mM $KHCO_3$, 0.1mM EDTA). After two washes in PBS to remove particulate matter the single cell suspension was aliquoted into ELISPOT plates previously coated with capture IFN-γ antibody and stimulated with CD8-restricted cognate peptide (Gag, Nef or RT). After overnight culture, IFN-γ producing cells were visualised by application of anti-murine IFN-γ-biotin labelled antibody (Pharmingen) followed by streptavidin -conjugated alkaline phosphatase and quantitated using image analysis.

The result of this experiment are shown in figures 20, 21, and 22.

Example 22:

Immunogenicity of Vaccine Constructs

1. Cellular Assays

The cellular immune response comprises cytotoxic CD8 cells and helper CD4 cells. A sensitive method to detect specific CD8 and CD4 cells is the ELIspot assay which can be used to quantify the number of cells capable of secreting interferon-γ or IL-2. The ELIspot assay relies on the capture of cytokines secreted from individual cells. Briefly, specialised microtitre plates are coated with anti-cytokine antibodies. Splenocytes isolated from immunised animals are incubated overnight in the presence of specific peptides representing known epitopes (CD8) or proteins (CD4). If cells are stimulated to release cytokines they will bind to the antibodies on the surface of the plate surrounding the locality of the individual producing cells. Cytokines remain attached to the coating antibody after the cells have been lysed and plates washed. The assay is developed in a similar way to an ELISA assay using a biotin/avidin amplification system. The number of spots equates to the number of cytokine producing cells.

CD8 responses to the following $K2^d$-restricted murine epitopes: Gag (AMQMLKETI), Nef (MTYKAAVDL) and RT (YYDPSKDLI) and CD4 responses to Gag and RT proteins were recorded for all 6 constructs. The results of these assays were analysed statistically and constructs were ranked according to their immunogenicity. The result is shown in figure 23 of the figures.

2. Humoral Assays

Blood samples were collected for antibody analysis at 7 and 14 days post-boost from two experiments. Serum was separated and stored frozen until antibody titres could be measured using specific ELISA assays. All samples were tested for antibodies to Gag, Nef and RT. Briefly, ELISA plates were coated with the relevant protein. Excess protein was washed off before diluted serum samples were incubated in the wells. The serum samples were washed off and anti-mouse antiserum conjugated to an appropriate tag was added. The plate was developed and read on a plate reader. The results are shown in figure 24.

3. Antibody Data

Antibody titres were measured for all six constructs in four experiments. Construct p73i-GNR consistently generated no antibody responses to Gag and limited antibody responses to Nef. The reason for this is unclear, as T-cell responses were observed from splenocytes isolated from the same mice, indicating that the Gag protein was being expressed *in vivo*.

The ranking for the generation of Gag specific antibodies was:

RNG>GRN>NRG>RGN>NGR>GNR

Analysis cellular immunology data

The objective was to rank the 6 constructs on the basis of spot count data from 3 immunology experiments. Three sets of responses were assessed:

CD8 responses to Gag, Nef and RT at Day 7 (7 days post primary),

CD4 responses to Gag and RT at Day 35 (7 days post boost),
CD8 responses to Gag, Nef and RT at Day 35 (7 days post boost).

Each response (e.g. CD8 response to Gag) was modeled using a linear mixed effect model in SAS version 8. The model included fixed effects of construct, whether the particular antigen (Gag, Nef or RT) was present or absent, and whether IL-2 was present or absent. In addition, for CD8 responses, where data were available from each individual mouse, subject was included as a random effect in the model. The model included interaction terms to allow for a different effect of construct for each combination of the antigen (present/absent) and IL-2 (present/absent).

From the model, the difference in adjusted mean response between each construct and p7313 (the control group) was estimated separately for each combination of antigen (present/absent) and IL-2 (present/absent), together with a p-value indicating whether the difference was statistically significant. Based on the differences and p-values in the presence of the antigen and the absence of IL-2, constructs were ranked, by assigning a score of 6 to the construct with the largest difference, 5 to the next largest, etc, but 0 to any constructs where the difference was not statistically significant at the 5% level.

The assumptions of the model – that the residuals were normally distributed with constant variance, were assessed using graphical methods and sensitivity analyses, where first a log and second a square root transformation of the response was modeled. The ranking

of the constructs was not sensitive to departures from the assumptions of the model.

Having calculated the ranks for each response in each experiment separately, total ranks for the 3 sets of responses were calculated across all 3 experiments. The following table shows the total rankings across the 3 experiments.

Total rankings of constructs for each of 3 sets of responses, combined across 3 immunology experiments.

Construct	**Day 7 (7 days post primary)**	**Day 35 (7 days post boost)**	
	CD8	CD4	CD8
GRN	5	18	3
GNR	17	24	28
RGN	**28**	23	33
RNG	25	**27**	**37**
NRG	25	19	0
NGR	4	14	10

RNG has the highest ranking for both sets of responses at Day 35, and the second highest ranking behind RGN at Day 7. RGN also receives high rankings for both sets of responses at Day 35.

CLAIMS

1. A nucleotide sequence that encodes an HIV-1 gag protein or fragment containing a gag epitope thereof and a second HIV antigen or a fragment encoding an epitope of said second HIV antigen, operably linked to a heterologous promoter.

2. A nucleotide sequence as claimed in claim 1 wherein the second antigen is selected from the group consisting of: Nef or a fragment thereof containing a nef epitope, or reverse transcriptase (RT) or a fragment thereof containing an RT epitope.

3. A nucleotide sequence as claimed in claim 1 or 2 wherein the gag protein comprises p17.

4. A nucleotide sequence as claimed in claim 3 wherein the gag protein additionally comprises p24.

5. A nucleotide sequence as claimed in any one of claims 1 to 4 wherein the gag sequence is codon optimised to resemble the codon usage in a highly expressed human gene.

6. A nucleotide sequence as claimed in any one of claims 1-5 wherein the sequence encodes an HIV-1 gag

protein or fragment containing a gag epitope, a nef protein or a fragment containing a nef epitope and an RT protein or a fragment containing an RT epitope, preferably in the order RT, Gag, Nef or RT, Nef, Gag.

7. A nucleotide sequence as claimed in any of claim 2-6 wherein the RT sequence or fragment thereof is codon optimised to resemble a highly expressed human gene.

8. A nucleotide sequence as claimed in any of claims 1 to 7 wherein the nucleotide sequence encodes a nef protein or epitope thereof.

9. A nucleotide sequence selected from the group consisting of:

- Gag (p17,p24), Nef truncate
- Gag (p17,p24) (codon optimised), Nef (truncate)
- Gag (p17,p24), RT, Nef (truncate)
- Gag (p17,p24) codon optimised, RT, Nef (truncate)
- Gag (p17,p24) codon optimised, RT codon optimised, Nef truncate
- RT (codon optimised), Gag (p17, p24) codon optimised, Nef truncate
- RT (codon optimised), Nef truncate, gag p17, p24 codon optimised

10. A nucleotide sequence as claimed in any of claims 1 to 9 wherein the heterologous promoter is the promoter from HCMV IE gene.

11. A nucleotide sequence as claimed in claim 10 wherein the 5' of the promoter comprises exon 1.

12. A vector comprising a nucleotide sequence as claimed in any one of claims 1 to 11.

13. A vector as claimed in claim 12 which is a double stranded DNA plasmid.

14. A pharmaceutical composition comprising a nucleotide sequence of claim 1 to 11 or a vector of claim 12 or 13 and a pharmaceutically acceptable excipient, diluent, carrier or adjuvant.

15. A pharmaceutical composition as claimed in claim 14 adapted for intra-muscular or intra-dermal delivery.

16. A pharmaceutical composition as claimed in claim 14 or 15 wherein the carrier is a gold bead.

17. An intra-dermal delivery device comprising a pharmaceutical composition of claim 14, 15 or 16.

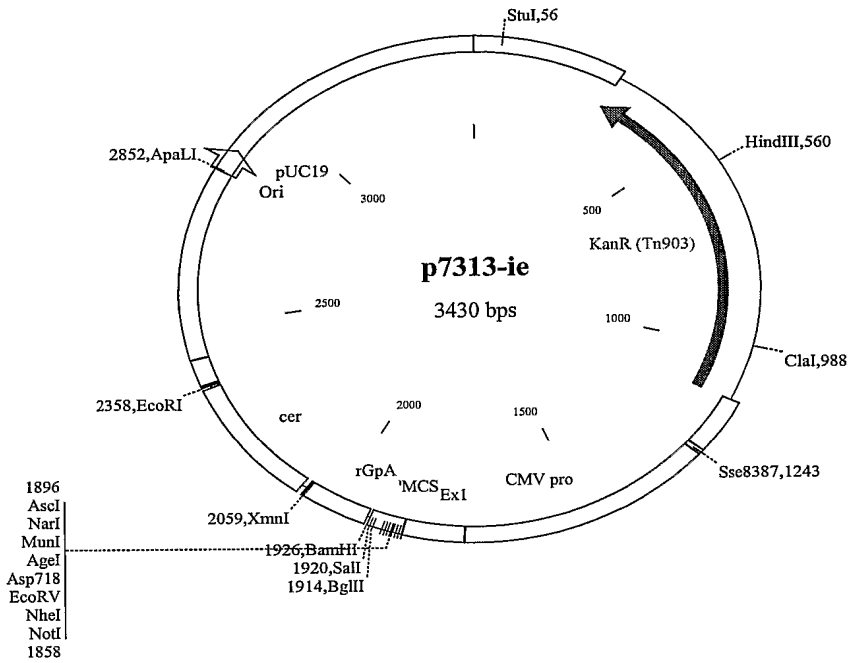
18. A method of treating a patient suffering from or susceptible to a disease comprising administration of a safe and effective amount of a pharmaceutical composition as claimed in any of claims 14 to 16.

19. A nucleotide sequence of any of claims 1 to 11, a vector of claim 12 or 13, or a composition of any of claims 14 to 16, for use in medicine.

20. Use of a nucleotide sequence of any of claims 1 to 11 in the manufacture of a medicament for the treatment of disease.

21. A process for the production of a nucleotide as claimed in any of claims 1 to 11 comprising operably linking a nucleotide sequence encoding an HIV-1 gag protein or fragment thereof and a second HIV protein or fragement thereof to a heterologous promoter sequence.

Figure 1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1000
1500
2000
2500
3000
2059,XmnI
1926,BamHI
1920,SalI
1914,BglII
1896
AscI
NarI
MunI
AgeI
Asp718
EcoRV
NheI
NotI
1858

Figure 2

Sequence of p55 gag insert in pGagOptrpr2

ATGGGTGCCCGAGCTTCGGTACTGTCTGGTGGAGAGCTGGACAGATGGGAGAAAATT
AGGCTGCGCCCGGGAGGCAAAAAGAAATACAAGCTCAAGCATATCGTGTGGGCCTCG
AGGGAGCTTGAACGGTTTGCCGTGAACCCAGGCCTGCTGGAAACATCTGAGGGATGT
CGCCAGATCCTGGGGCAATTGCAGCCATCCCTCCAGACCGGGAGTGAAGAGCTGAGG
TCCTTGTATAACACAGTGGCTACCCTCTACTGCGTACACCAGAGGATCGAGATTAAG
GATACCAAGGAGGCCTTGGACAAAATTGAGGAGGAGCAAAACAAGAGCAAGAAGAAG
GCCCAGCAGGCAGCTGCTGACACTGGGCATAGCAACCAGGTATCACAGAACTATCCT
ATTGTCCAAAACATTCAGGGCCAGATGGTTCATCAGGCCATCAGCCCCCGGACGCTC
AATGCCTGGGTGAAGGTTGTCGAAGAGAAGGCCTTTTCTCCTGAGGTTATCCCCATG
TTCTCCGCTTTGAGTGAGGGGGCCACTCCTCAGGACCTCAATACAATGCTTAATACC
GTGGGCGGCCATCAGGCCGCCATGCAAATGTTGAAGGAGACTATCAACGAGGAGGCA
GCCGAGTGGGACAGAGTGCATCCCGTCCACGCTGGCCCAATCGCGCCCGGACAGATG
CGGGAGCCTCGCGGCTCTGACATTGCCGGCACCACCTCTACACTGCAAGAGCAAATC
GGATGGATGACCAACAATCCTCCCATCCCAGTTGGAGAAATCTATAAACGGTGGATC
ATTCTCGGTCTCAATAAAATTGTTAGAATGTACTCTCCGACATCCATCCTTGACATT
AGACAGGGACCCAAAGAGCCTTTTAGGGATTACGTCGACCGGTTTTATAAGACCCTG
CGAGCAGAGCAGGCCTCTCAGGAGGTCAAAAACTGGATGACGGAGACACTCCTGGTA
CAGAACGCTAACCCCGACTGCAAAACAATCTTGAAGGCACTAGGCCCGGCTGCCACC
CTGGAAGAGATGATGACCGCCTGTCAGGGAGTAGGCGGACCCGGACACAAAGCCAGA
GTGTTGGCCGAAGCCATGAGCCAGGTGACGAACTCCGCAACCATCATGATGCAGAGA
GGGAACTTCCGCAATCAGCGGAAGATCGTGAAGTGTTTCAATTGCGGCAAGGAGGGT
CATACCGCCCGCAACTGTCGGGCCCCTAGGAAGAAAGGGTGTTGGAAGTGCGGCAAG
GAGGGACACCAGATGAAAGACTGTACAGAACGACAGGCCAATTTTCTTGGAAAGATT
TGGCCGAGCTACAAGGGGAGACCTGGTAATTTCCTGCAAAGCAGGCCCGAGCCCACC
GCCCCCCCTGAGGAATCCTTCAGGTCCGGAGTGGAGACCACAACGCCTCCCCAAAAA
CAGGAACCAATCGACAAGGAGCTGTACCCTTTAACTTCTCTGCGTTCTCTCTTTGGC
AACGACCCGTCGTCTCAATAA [SEQ ID NO: 50]

MGARASVLSG GELDRWEKIR LRPGGKKKYK LKHIVWASRE LERFAVNPGL
LETSEGCRQI LGQLQPSLQT GSEELRSLYN TVATLYCVHQ RIEIKDTKEA
LDKIEEEQNK SKKKAQQAAA DTGHSNQVSQ NYPIVQNIQG QMVHQAISPR
TLNAWVKVVE EKAFSPEVIP MFSALSEGAT PQDLNTMLNT VGGHQAAMQM
LKETINEEAA EWDRVHPVHA GPIAPGQMRE PRGSDIAGTT STLQEQIGWM
TNNPPIPVGE IYKRWIILGL NKIVRMYSPT SILDIRQGPK EPFRDYVDRF
YKTLRAEQAS QEVKNWMTET LLVQNANPDC KTILKALGPA ATLEEMMTAC
QGVGGPGHKA RVLAEAMSQV TNSATIMMQR GNFRNQRKIV KCFNCGKEGH
TARNCRAPRK KGCWKCGKEG HQMKDCTERQ ANFLGKIWPS YKGRPGNFLQ
SRPEPTAPPE ESFRSGVETT TPPQKQEPID KELYPLTSLR SLFGNDPSSQ
*
[SEQ ID NO: 51]

Figure 3

Sequence of the p17/24trNEF insert in p17/24trNEF1

ATGGGTGCGAGAGCGTCAGTATTAAGCGGGGGAGAATTAGATCGATGGGAAAAAATT
CGGTTAAGGCCAGGGGGAAAGAAAAAATATAAATTAAAACATATAGTATGGGCAAGC
AGGGAGCTAGAACGATTCGCAGTTAATCCTGGCCTGTTAGAAACATCAGAAGGCTGT
AGACAAATACTGGGACAGCTACAACCATCCCTTCAGACAGGATCAGAAGAACTTAGA
TCATTATATAATACAGTAGCAACCCTCTATTGTGTGCATCAAAGGATAGAGATAAAA
GACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAGAAAAAA
GCACAGCAAGCAGCAGCTGACACAGGACACAGCAATCAGGTCAGCCAAAATTACCCT
ATAGTGCAGAACATCCAGGGGCAAATGGTACATCAGGCCATATCACCTAGAACTTTA
AATGCATGGGTAAAAGTAGTAGAAGAGAAGGCTTTCAGCCCAGAAGTGATACCCATG
TTTTCAGCATTATCAGAAGGAGCCACCCCACAAGATTTAAACACCATGCTAAACACA
GTGGGGGGACATCAAGCAGCCATGCAAATGTTAAAAGAGACCATCAATGAGGAAGCT
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GGATGGATGACAAATAATCCACCTATCCCAGTAGGAGAAATTTATAAAAGATGGATA
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AGACAAGGACCAAAAGAACCCTTTAGAGACTATGTAGACCGGTTCTATAAAACTCTA
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CAAAATGCGAACCCAGATTGTAAGACTATTTTAAAAGCATTGGGACCAGCGGCTACA
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GTTTTGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATGACTTACAAG
GCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGGGGACTGGAAGGGCTAATT
CACTCCCAAAGAAGACAAGATATCCTTGATCTGTGGATCTACCACACACAAGGCTAC
TTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCAGATATCCACTGACCTTT
GGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAGGCCAATAAA
GGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGGATGGATGACCCGGAG
AGAGAAGTGTTAGAGTGGAGGTTTGACAGCC**A**CCTAGCATTTCATCACGTGGCCCGA
GAGCTGCATCCGGAGTACTTCAAGAACTGCTGA [SEQ ID NO: 52]

MGARASVLSG GELDRWEKIR LRPGGKKKYK LKHIVWASRE LERFAVNPGL
LETSEGCRQI LGQLQPSLQT GSEELRSLYN TVATLYCVHQ RIEIKDTKEA
LDKIEEEQNK SKKKAQQAAA DTGHSNQVSQ NYPIVQNIQG QMVHQAISPR
TLNAWVKVVE EKAFSPEVIP MFSALSEGAT PQDLNTMLNT VGGHQAAMQM
LKETINEEAA EWDRVHPVHA GPIAPGQMRE PRGSDIAGTT STLQEQIGWM
TNNPPIPVGE IYKRWIILGL NKIVRMYSPT SILDIRQGPK EPFRDYVDRF
YKTLRAEQAS QEVKNWMTET LLVQNANPDC KTILKALGPA ATLEEMMTAC
QGVGGPGHKA RVLVGFPVTP QVPLRPMTYK AAVDLSHFLK EKGGLEGLIH
SQRRQDILDL WIYHTQGYFP DWQNYTPGPG VRYPLTFGWC YKLVPVEPDK
VEEANKGENT SLLHPVSLHG MDDPEREVLE WRFDS**H**LAFH HVARELHPEY
FKNC*
[SEQ ID NO: 53]

Figure 4

Sequence of the p17/24opt/trNef insert in p17/24opt/trNef1

ATGGGTGCCCGAGCTTCGGTACTGTCTGGTGGAGAGCTGGACAGATGGGAGAAAATT
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CGCCAGATCCTGGGGCAATTGCAGCCATCCCTCCAGACCGGGAGTGAAGAGCTGAGG
TCCTTGTATAACACAGTGGCTACCCTCTACTGCGTACACCAGAGGATCGAGATTAAG
GATACCAAGGAGGCCTTGGACAAAATTGAGGAGGAGCAAAACAAGAGCAAGAAGAAG
GCCCAGCAGGCAGCTGCTGACACTGGGCATAGCAACCAGGTATCACAGAACTATCCT
ATTGTCCAAAACATTCAGGGCCAGATGGTTCATCAGGCCATCAGCCCCCGGACGCTC
AATGCCTGGGTGAAGGTTGTCGAAGAGAAGGCCTTTTCTCCTGAGGTTATCCCCATG
TTCTCCGCTTTGAGTGAGGGGGCCACTCCTCAGGACCTCAATACAATGCTTAATACC
GTGGGCGGCCATCAGGCCGCCATGCAAATGTTGAAGGAGACTATCAACGAGGAGGCA
GCCGAGTGGGACAGAGTGCATCCCGTCCACGCTGGCCCAATCGCGCCCGGACAGATG
CGGGAGCCTCGCGGCTCTGACATTGCCGGCACCACCTCTACACTGCAAGAGCAAATC
GGATGGATGACCAACAATCCTCCCATCCCAGTTGGAGAAATCTATAAACGGTGGATC
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CGAGCAGAGCAGGCCTCTCAGGAGGTCAAAAACTGGATGACGGAGACACTCCTGGTA
CAGAACGCTAACCCCGACTGCAAAACAATCTTGAAGGCACTAGGCCCGGCTGCCACC
CTGGAAGAGATGATGACCGCCTGTCAGGGAGTAGGCGGACCCGGACACAAAGCCAGA
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TACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCAGATATCCACTGACC
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GAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCACCTAGCATTTCATCACGTGGCC
CGAGAGCTGCATCCGGAGTACTTCAAGAACTGCTGA [SEQ ID NO: 54]

MGARASVLSG GELDRWEKIR LRPGGKKKYK LKHIVWASRE LERFAVNPGL
LETSEGCRQI LGQLQPSLQT GSEELRSLYN TVATLYCVHQ RIEIKDTKEA
LDKIEEEQNK SKKKAQQAAA DTGHSNQVSQ NYPIVQNIQG QMVHQAISPR
TLNAWVKVVE EKAFSPEVIP MFSALSEGAT PQDLNTMLNT VGGHQAAMQM
LKETINEEAA EWDRVHPVHA GPIAPGQMRE PRGSDIAGTT STLQEQIGWM
TNNPPIPVGE IYKRWIILGL NKIVRMYSPT SILDIRQGPK EPFRDYVDRF
YKTLRAEQAS QEVKNWMTET LLVQNANPDC KTILKALGPA ATLEEMMTAC
QGVGGPGHKA RVLMVGFPVT PQVPLRPMTY KAAVDLSHFL KEKGGLEGLI
HSQRRQDILD LWIYHTQGYF PDWQNYTPGP GVRYPLTFGW CYKLVPVEPD
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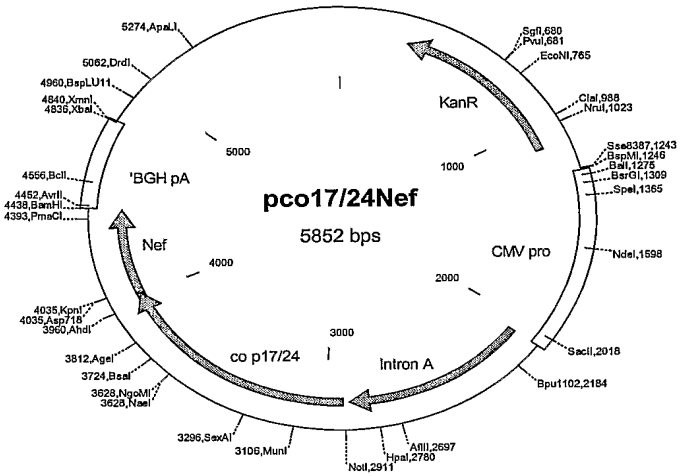
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BalI,1275
BsrGI,1309
SpeI,1365
NdeI,1598
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Bpu1102,2184
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HpaI,2780
NotI,2911
3106,MunI
3296,SexAI
3628,NaeI
3628,NgoMI
3724,BseI
3812,AgeI
3960,AhdI
4035,Asp718
4035,KpnI
4393,PmaCI
4438,BamHI
4452,AvrII
4556,BclI
4835,XbaI
4840,XmnI
4960,BspLU11I
5062,DrdI
5274,ApaLI

Figure 5

Sequence of RT insert of p7077-RT3:

ATGGGCCCCATCAGTCCCATCGAGACCGTGCCGGTGAAGCTGAAACCCGGGATGGAC
GGCCCCAAGGTCAAGCAGTGGCCACTCACCGAGGAGAAGATCAAGGCCCTGGTGGAG
ATCTGCACCGAGATGGAGAAAGAGGGCAAGATCAGCAAGATCGGGCCTGAGAACCCA
TACAACACCCCCGTGTTTGCCATCAAGAAGAAGGACAGCACCAAGTGGCGCAAGCTG
GTGGATTTCCGGGAGCTGAATAAGCGGACCCAGGATTTCTGGGAGGTCCAGCTGGGC
ATCCCCCATCCGGCCGGCCTGAAGAAGAAGAAGAGCGTGACCGTGCTGGACGTGGGC
GACGCTTACTTCAGCGTCCCTCTGGACGAGGACTTTAGAAAGTACACCGCCTTTACC
ATCCCATCTATCAACAACGAGACCCCTGGCATCAGATATCAGTACAACGTCCTCCCC
CAGGGCTGGAAGGGCTCTCCCGCCATTTTCCAGAGCTCCATGACCAAGATCCTGGAG
CCGTTTCGGAAGCAGAACCCCGATATCGTCATCTACCAGTACATGGACGACCTGTAC
GTGGGCTCTGACCTGGAAATCGGGCAGCATCGCACGAAGATTGAGGAGCTGAGGCAG
CATCTGCTGAGATGGGGCCTGACCACTCCGGACAAGAAGCATCAGAAGGAGCCGCCA
TTCCTGTGGATGGGCTACGAGCTCCATCCCGACAAGTGGACCGTGCAGCCTATCGTC
CTCCCCGAGAAGGACAGCTGGACCGTGAACGACATCCAGAAGCTGGTGGGCAAGCTC
AACTGGGCTAGCCAGATCTATCCCGGGATCAAGGTGCGCCAGCTCTGCAAGCTGCTG
CGCGGCACCAAGGCCCTGACCGAGGTGATTCCCCTCACGGAGGAAGCCGAGCTCGAG
CTGGCTGAGAACCGGGAGATCCTGAAGGAGCCCGTGCACGGCGTGTACTATGACCCC
TCCAAGGACCTGATCGCCGAAATCCAGAAGCAGGGCCAGGGGCAGTGGACATACCAG
ATTTACCAGGAGCCTTTCAAGAACCTCAAGACCGGCAAGTACGCCCGCATGAGGGGC
GCCCACACCAACGATGTCAAGCAGCTGACCGAGGCCGTCCAGAAGATCACGACCGAG
TCCATCGTGATCTGGGGGAAGACACCCAAGTTCAAGCTGCCTATCCAGAAGGAGACC
TGGGAGACGTGGTGGACCGAATATTGGCAGGCCACCTGGATTCCCGAGTGGGAGTTC
GTGAATACACCTCCTCTGGTGAAGCTGTGGTACCAGCTCGAGAAGGAGCCCATCGTG
GGCGCGGAGACATTCTACGTGGACGGCGCGGCCAACCGCGAAACAAAGCTCGGGAAG
GCCGGGTACGTCACCAACCGGGGCCGCCAGAAGGTCGTCACCCTGACCGACACCACC
AACCAGAAGACGGAGCTGCAGGCCATCTATCTCGCTCTCCAGGACTCCGGCCTGGAG
GTGAACATCGTGACGGACAGCCAGTACGCGCTGGGCATTATTCAGGCCCAGCCGGAC
CAGTCCGAGAGCGAACTGGTGAACCAGATTATCGAGCAGCTGATCAAGAAAGAGAAG
GTCTACCTCGCCTGGGTCCCGGCCCATAAGGGCATTGGCGGCAACGAGCAGGTCGAC
AAGCTGGTGAGTGCGGGGATTAGAAAGGTGCTGTAA [SEQ ID NO: 56]

MGPISPIETV SVKLKPGMDG PKVKQWPLTE EKIKALVEIC TEMEKEGKIS
KIGPENPYNT PVFAIKKKDS TKWRKLVDFR ELNKRTQDFW EVQLGIPHPA
GLKKKKSVTV LDVGDAYFSV PLDEDFRKYT AFTIPSINNE TPGIRYQYNV
LPQGWKGSPA IFQSSMTKIL EPFRKQNPDI VIYQYMDDLY VGSDLEIGQH
RTKIEELRQH LLRWGLTTPD KKHQKEPPFL WMGYELHPDK WTVQPIVLPE
KDSWTVNDIQ KLVGKLNWAS QIYPGIKVRQ LCKLLRGTKA LTEVIPLTEE
AELELAENRE ILKEPVHGVY YDPSKDLIAE IQKQGQGQWT YQIYQEPFKN
LKTGKYARMR GAHTNDVKQL TEAVQKITTE SIVIWGKTPK FKLPIQKETW
ETWWTEYWQA TWIPEWEFVN TPPLVKLWYQ LEKEPIVGAE TFYVDGAANR
ETKLGKAGYV TNRGRQKVVT LTDTTNQKTE LQAIYLALQD SGLEVNIVTD
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DKLVSAGIRK VL*

[SEQ ID NO: 57]

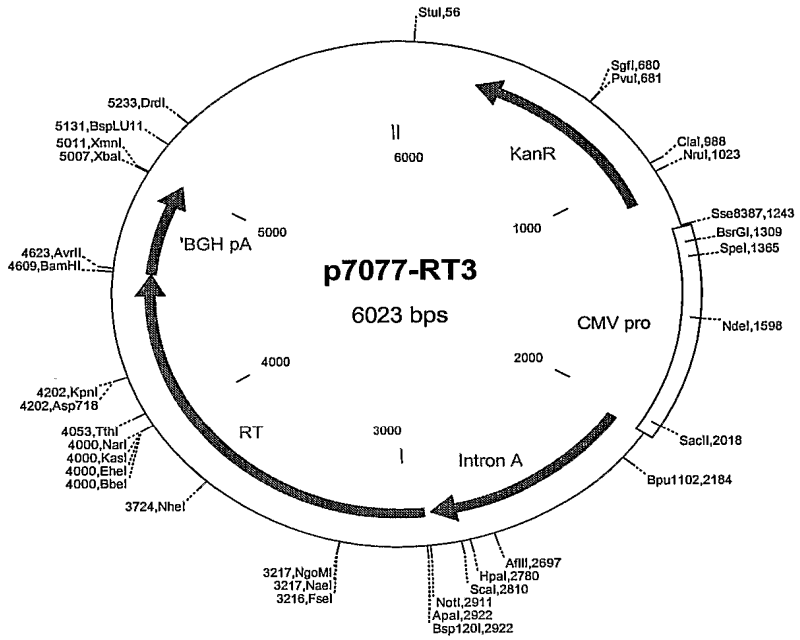

Figure 6

Sequence of the coding insert in p73i-RT3:

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ATCTGCACCGAGATGGAGAAAGAGGGCAAGATCAGCAAGATCGGGCCTGAGAACCCA
TACAACACCCCCGTGTTTGCCATCAAGAAGAAGGACAGCACCAAGTGGCGCAAGCTG
GTGGATTTCCGGGAGCTGAATAAGCGGACCCAGGATTTCTGGGAGGTCCAGCTGGGC
ATCCCCCATCCGGCCGGCCTGAAGAAGAAGAAGAGCGTGACCGTGCTGGACGTGGGC
GACGCTTACTTCAGCGTCCCTCTGGACGAGGACTTTAGAAAGTACACCGCCTTTACC
ATCCCATCTATCAACAACGAGACCCCTGGCATCAGATATCAGTACAACGTCCTCCCC
CAGGGCTGGAAGGGCTCTCCCGCCATTTTCCAGAGCTCCATGACCAAGATCCTGGAG
CCGTTTCGGAAGCAGAACCCCGATATCGTCATCTACCAGTACATGGACGACCTGTAC
GTGGGCTCTGACCTGGAAATCGGGCAGCATCGCACGAAGATTGAGGAGCTGAGGCAG
CATCTGCTGAGATGGGGCCTGACCACTCCGGACAAGAAGCATCAGAAGGAGCCGCCA
TTCCTGTGGATGGGCTACGAGCTCCATCCCGACAAGTGGACCGTGCAGCCTATCGTC
CTCCCCGAGAAGGACAGCTGGACCGTGAACGACATCCAGAAGCTGGTGGGCAAGCTC
AACTGGGCTAGCCAGATCTATCCCGGGATCAAGGTGCGCCAGCTCTGCAAGCTGCTG
CGCGGCACCAAGGCCCTGACCGAGGTGATTCCCCTCACGGAGGAAGCCGAGCTCGAG
CTGGCTGAGAACCGGGAGATCCTGAAGGAGCCCGTGCACGGCGTGTACTATGACCCC
TCCAAGGACCTGATCGCCGAAATCCAGAAGCAGGGCCAGGGGCAGTGGACATACCAG
ATTTACCAGGAGCCTTTCAAGAACCTCAAGACCGGCAAGTACGCCCGCATGAGGGGC
GCCCACACCAACGATGTCAAGCAGCTGACCGAGGCCGTCCAGAAGATCACGACCGAG
TCCATCGTGATCTGGGGGAAGACACCCAAGTTCAAGCTGCCTATCCAGAAGGAGACC
TGGGAGACGTGGTGGACCGAATATTGGCAGGCCACCTGGATTCCCGAGTGGGAGTTC
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GGCGCGGAGACATTCTACGTGGACGGCGCGGCCAACCGCGAAACAAAGCTCGGGAA
GGCCGGGTACGTCACCAACCGGGGCCGCCAGAAGGTCGTCACCCTGACCGACACCAC
CAACCAGAAGACGGAGCTGCAGGCCATCTATCTCGCTCTCCAGGACTCCGGCCTGGA
GGTGAACATCGTGACGGACAGCCAGTACGCGCTGGGCATTATTCAGGCCCAGCCGGA
CCAGTCCGAGAGCGAACTGGTGAACCAGATTATCGAGCAGCTGATCAAGAAAGAGAA
GGTCTACCTCGCCTGGGTCCCGGCCCATAAGGGCATTGGCGGCAACGAGCAGGTCGA
CAAGCTGGTGAGTGCGGGGATTAGAAAGGTGCTGTAA [SEQ ID NO: 58]

MGPISPIETV SVKLKPGMDG PKVKQWPLTE EKIKALVEIC TEMEKEGKIS
KIGPENPYNT PVFAIKKKDS TKWRKLVDFR ELNKRTQDFW EVQLGIPHPA
GLKKKKSVTV LDVGDAYFSV PLDEDFRKYT AFTIPSINNE TPGIRYQYNV
LPQGWKGSPA IFQSSMTKIL EPFRKQNPDI VIYQYMDDLY VGSDLEIGQH
RTKIEELRQH LLRWGLTTPD KKHQKEPPFL WMGYELHPDK WTVQPIVLPE
KDSWTVNDIQ KLVGKLNWAS QIYPGIKVRQ LCKLLRGTKA LTEVIPLTEE
AELELAENRE ILKEPVHGVY YDPSKDLIAE IQKQGQGQWT YQIYQEPFKN
LKTGKYARMR GAHTNDVKQL TEAVQKITTE SIVIWGKTPK FKLPIQKETW
ETWWTEYWQA TWIPEWEFVN TPPLVKLWYQ LEKEPIVGAE TFYVDGAANR
ETKLGKAGYV TNRGRQKVVT LTDTTNQKTE LQAIYLALQD SGLEVNIVTD
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DKLVSAGIRK VL*
[SEQ ID NO: 59]

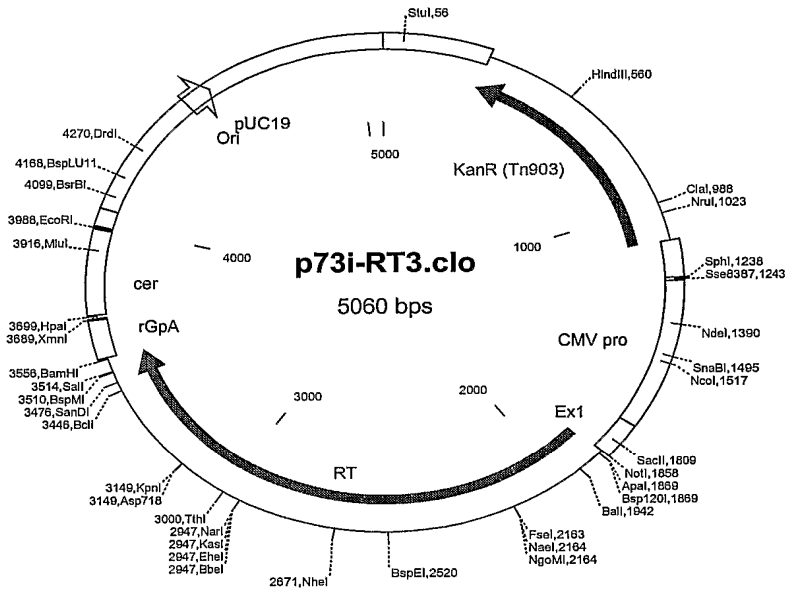

Figure 7

Sequence of Nef insert in 7077trNef20

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TCCCAAAGAAGACAAGATATCCTTGATCTGTGGATCTACCACACACAAGGCTACTTC
CCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCAGATATCCACTGACCTTTGGA
TGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAGGCCAATAAAGGA
GAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGGATGGATGACCCGGAGAGA
GAAGTGTTAGAGTGGAGGTTTGACAGCC**A**CCTAGCATTTCATCACGTGGCCCGAGAG
CTGCATCCGGAGTACTTCAAGAACTGCTGA [SEQ ID NO: 60]

MVGFPVTPQV PLRPMTYKAA VDLSHFLKEK GGLEGLIHSQ RRQDILDLWI
YHTQGYFPDW QNYTPGPGVR YPLTFGWCYK LVPVEPDKVE EANKGENTSL
LHPVSLHGMD DPEREVLEWR FDS**V**LAFHHV ARELHPEYFK NC*
[SEQ ID NO: 61]

Figure 8

Sequence of RT insert in 7077RT8

ATGGGCCCCATTAGCCCTATTGAGACTGTGTCAGTAAAATTAAAGCCAGGAATGGAT
GGCCCAAAAGTTAAACAATGGCCATTGACAGAAGAAAAAATAAAAGCATTAGTAGAA
ATTTGTACAGAGATGGAAAAGGAAGGGAAAATTTCAAAAATTGGGCCTGAAAATCCA
TACAATACTCCAGTATTTGCCATAAAGAAAAAAGACAGTACTAAATGGAGAAAATTA
GTAGATTTCAGAGAACTTAATAAGAGAACTCAAGACTTCTGGGAAGTTCAATTAGGA
ATACCACATCCCGCAGGGTTAAAAAAGAAAAAATCAGTAACAGTACTGGATGTGGGT
GATGCATATTTTTCAGTTCCCTTAGATGAAGACTTCAGGAAATATACTGCATTTACC
ATACCTAGTATAAACAATGAGACACCAGGGATTAGATATCAGTACAATGTGCTTCCA
CAGGGATGGAAAGGATCACCAGCAATATTCCAAAGTAGCATGACAAAAATCTTAGAG
CCTTTTAGAAAACAAAATCCAGACATAGTTATCTATCAATACATGGATGATTTGTAT
GTAGGATCTGACTTAGAAATAGGGCAGCATAGAACAAAAATAGAGGAGCTGAGACAA
CATCTGTTGAGGTGGGGACTTACCACACCAGACAAAAAACATCAGAAAGAACCTCCA
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CTGCCAGAAAAAGACAGCTGGACTGTCAATGACATACAGAAGTTAGTGGGGAAATTG
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GCCCACACTAATGATGTAAAACAATTAACAGAGGCAGTGCAAAAAATAACCACAGAA
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TGGGAAACATGGTGGACAGAGTATTGGCAAGCCACCTGGATTCCTGAGTGGGAGTTT
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GGAGCAGAAACCTTCTATGTAGATGGGGCAGCTAACAGGGAGACTAAATTAGGAAAA
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AATCAGAAGACTGAGTTACAAGCAATTTATCTAGCTTTGCAGGATTCGGGATTAGAA
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CAAAGTGAATCAGAGTTAGTCAATCAAATAATAGAGCAGTTAATAAAAAAGGAAAAG
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AAATTAGTCAGTGCTGGAATCAGGAAAGTACTATTTTTAGATTAA
[SEQ ID NO: 62]

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KIGPENPYNT PVFAIKKKDS TKWRKLVDFR ELNKRTQDFW EVQLGIPHPA
GLKKKKSVTV LDVGDAYFSV PLDEDFRKYT AFTIPSINNE TPGIRYQYNV
LPQGWKGSPA IFQSSMTKIL EPFRKQNPDI VIYQYMDDLY VGSDLEIGQH
RTKIEELRQH LLRWGLTTPD KKHQKEPPFL WMGYELHPDK WTVQPIVLPE
KDSWTVNDIQ KLVGKLNWAS QIYPGIKVRQ LCKLLRGTKA LTEVIPLTEE
AELELAENRE ILKEPVHGVY YDPSKDLIAE IQKQGQGQWT YQIYQEPFKN
LKTGKYARMR GAHTNDVKQL TEAVQKITTE SIVIWGKTPK FKLPIQKETW
ETWWTEYWQA TWIPEWEFVN TPPLVKLWYQ LEKEPIVGAE TFYVDGAANR
ETKLGKAGYV TNRGRQKVVT LTDTTNQKTE LQAIYLALQD SGLEVNIVTD
SQYALGIIQA QPDQSESELV NQIIEQLIKK EKVYLAWVPA HKGIGGNEQV
DKLVSAGIRK VLFLD*

[SEQ ID NO: 63]

Figure 9

Sequence of the p17/24opt/RT/trNef insert in p17/24opt/RT/trNef13

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CGCCAGATCCTGGGGCAATTGCAGCCATCCCTCCAGACCGGGAGTGAAGAGCTGAGG
TCCTTGTATAACACAGTGGCTACCCTCTACTGCGTACACCAGAGGATCGAGATTAAG
GATACCAAGGAGGCCTTGGACAAAATTGAGGAGGAGCAAAACAAGAGCAAGAAGAAG
GCCCAGCAGGCAGCTGCTGACACTGGGCATAGCAACCAGGTATCACAGAACTATCCT
ATTGTCCAAAACATTCAGGGCCAGATGGTTCATCAGGCCATCAGCCCCCGGACGCTC
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CGGGAGCCTCGCGGCTCTGACATTGCCGGCACCACCTCTACACTGCAAGAGCAAATC
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GTGGGTGATGCATATTTTTCAGTTCCCTTAGATGAAGACTTCAGGAAATATACTGCA
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TLNAWVKVVE EKAFSPEVIP MFSALSEGAT PQDLNTMLNT VGGHQAAMQM
LKETINEEAA EWDRVHPVHA GPIAPGQMRE PRGSDIAGTT STLQEQIGWM
TNNPPIPVGE IYKRWIILGL NKIVRMYSPT SILDIRQGPK EPFRDYVDRF
YKTLRAEQAS QEVKNWMTET LLVQNANPDC KTILKALGPA ATLEEMMTAC
QGVGGPGHKA RVLMGPISPI ETVSVKLKPG MDGPKVKQWP LTEEKIKALV
EICTEMEKEG KISKIGPENP YNTPVFAIKK KDSTKWRKLV DFRELNKRTQ
DFWEVQLGIP HPAGLKKKKS VTVLDVGDAY FSVPLDEDFR KYTAFTIPSI
NNETPGIRYQ YNVLPQGWKG SPAIFQSSMT KILEPFRKQN PDIVIYQYMD
DLYVGSDLEI GQHRTKIEEL RQHLLRWGLT TPDKKHQKEP PFLWMGYELH
PDKWTVQPIV LPEKDSWTVN DIQKLVGKLN WASQIYPGIK VRQLCKLLRG
TKALTEVIPL TEEAELELAE NREILKEPVH GVYYDPSKDL IAEIQKQGQG
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TPKFKLPIQK ETWETWWTEY WQATWIPEWE FVNTPPLVKL WYQLEKEPIV
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VPAHKGIGGN EQVDKLVSAG IRKVLFLDMV GFPVTPQVPL RPMTYKAAVD
LSHFLKEKGG LEGLIHSQRR QDILDLWIYH TQGYFPDWQN YTPGPGVRYP
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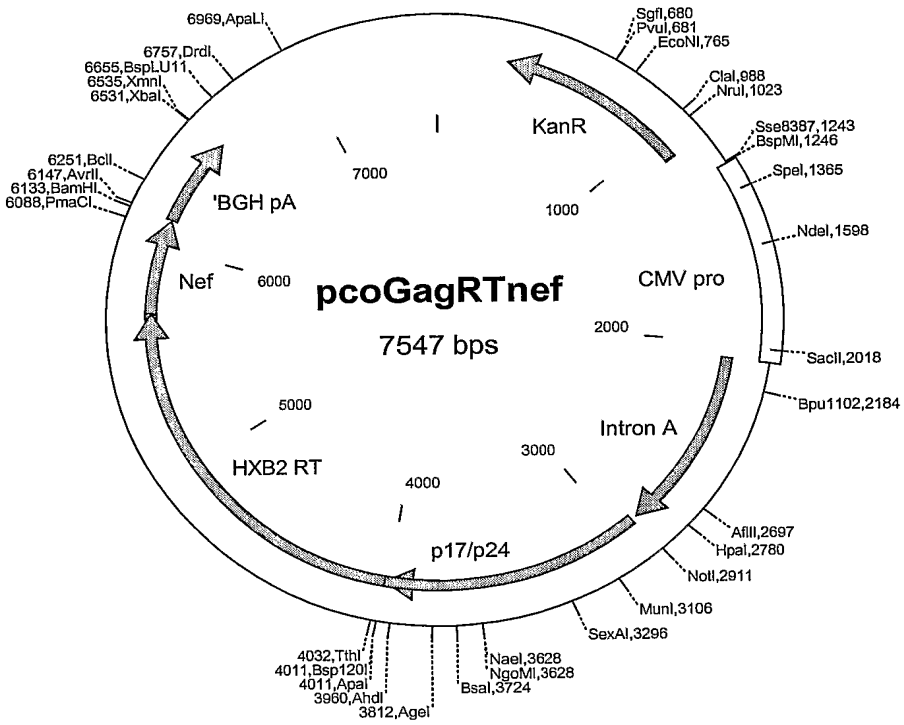
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4011,Bsp120I
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3960,AhdI
3812,AgeI
6969,ApaLI
6757,DrdI
6655,BspLU11I
6535,XmnI
6531,XbaI
6251,BclI
6147,AvrII
6133,BamHI
6088,PmaCI

Figure 10

Sequence of the p17/p24opt(cor)/RT/trNef coding insert in WRG7077:

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GATACCAAGGAGGCCTTGGACAAAATTGAGGAGGAGCAAAACAAGAGCAAGAAGAAG
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ATTGTCCAAAACATTCAGGGCCAGATGGTTCATCAGGCCATCAGCCCCCGGACGCTC
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TTCTCCGCTTTGAGTGAGGGGGCCACTCCTCAGGACCTCAATACAATGCTTAATACC
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ACAACAAATCAGAAGACTGAGTTACAAGCAATTTATCTAGCTTTGCAGGATTCGGGA
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TLNAWVKVVE EKAFSPEVIP MFSALSEGAT PQDLNTMLNT VGGHQAAMQM
LKETINEEAA EWDRVHPVHA GPIAPGQMRE PRGSDIAGTT STLQEQIGWM
TNNPPIPVGE IYKRWIILGL NKIVRMYSPT SILDIRQGPK EPFRDYVDRF
YKTLRAEQAS QEVKNWMTET LLVQNANPDC KTILKALGPA ATLEEMMTAC
QGVGGPGHKA RVLMGPISPI ETVSVKLKPG MDGPKVKQWP LTEEKIKALV
EICTEMEKEG KISKIGPENP YNTPVFAIKK KDSTKWRKLV DFRELNKRTQ
DFWEVQLGIP HPAGLKKKKS VTVLDVGDAY FSVPLDEDFR KYTAFTIPSI
NNETPGIRYQ YNVLPQGWKG SPAIFQSSMT KILEPFRKQN PDIVIYQYMD
DLYVGSDLEI GQHRTKIEEL RQHLLRWGLT TPDKKHQKEP PFLWMGYELH
PDKWTVQPIV LPEKDSWTVN DIQKLVGKLN WASQIYPGIK VRQLCKLLRG
TKALTEVIPL TEEAELELAE NREILKEPVH GVYYDPSKDL IAEIQKQGQG
QWTYQIYQEP FKNLKTGKYA RMRGAHTNDV KQLTEAVQKI TTESIVIWGK
TPKFKLPIQK ETWETWWTEY WQATWIPEWE FVNTPPLVKL WYQLEKEPIV
GAETFYVDGA ANRETKLGKA GYVTNRGRQK VVTLTDTTNQ KTELQAIYLA
LQDSGLEVNI VTDSQYALGI IQAQPDQSES ELVNQIIEQL IKKEKVYLAW
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LSHFLKEKGG LEGLIHSQRR QDILDLWIYH TQGYFPDWQN YTPGPGVRYP
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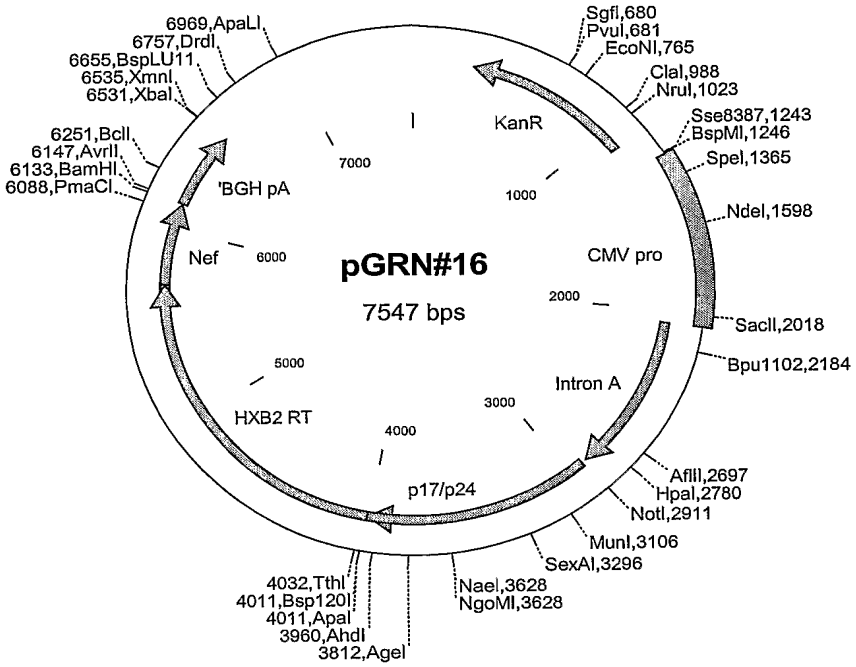
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KanR
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EcoNI,765
ClaI,988
NruI,1023
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NotI,2911
MunI,3106
SexAI,3296
NaeI,3628
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6655,BspLU11
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6531,XbaI
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6133,BamHI
6088,PmaCI

Figure 11

Sequence of the p17/p24(opt)/RT(opt)trNef insert in p73i-GRN2:

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GAGGTTATCCCCATGTTCTCCGCTTTGAGTGAGGGGGCCACTCCTCAGGAC
CTCAATACAATGCTTAATACCGTGGGCGGCCATCAGGCCGCCATGCAAAT
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CCCGTCCACGCTGGCCCAATCGCGCCCGGACAGATGCGGGAGCCTCGCGG
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EICTEMEKEG KISKIGPENP YNTPVFAIKK KDSTKWRKLV DFRELNKRTQ
DFWEVQLGIP HPAGLKKKKS VTVLDVGDAY FSVPLDEDFR KYTAFTIPSI
NNETPGIRYQ YNVLPQGWKG SPAIFQSSMT KILEPFRKQN PDIVIYQYMD
DLYVGSDLEI GQHRTKIEEL RQHLLRWGLT TPDKKHQKEP PFLWMGYELH
PDKWTVQPIV LPEKDSWTVN DIQKLVGKLN WASQIYPGIK VRQLCKLLRG
TKALTEVIPL TEEAELELAE NREILKEPVH GVYYDPSKDL IAEIQKQGQG
QWTYQIYQEP FKNLKTGKYA RMRGAHTNDV KQLTEAVQKI TTESIVIWGK
TPKFKLPIQK ETWETWWTEY WQATWIPEWE FVNTPPLVKL WYQLEKEPIV
GAETFYVDGA ANRETKLGKA GYVTNRGRQK VVTLTDTTNQ KTELQAIYLA
LQDSGLEVNI VTDSQYALGI IQAQPDQSES ELVNQIIEQL IKKEKVYLAW
VPAHKGIGGN EQVDKLVSAG IRKVLMVGFP VTPQVPLRPM TYKAAVDLSH
FLKEKGGLEG LIHSQRRQDI LDLWIYHTQG YFPDWQNYTP GPGVRYPLTF
GWCYKLVPVE PDKVEEANKG ENTSLLHPVS LHGMDDPERE VLEWRFDS**R**L
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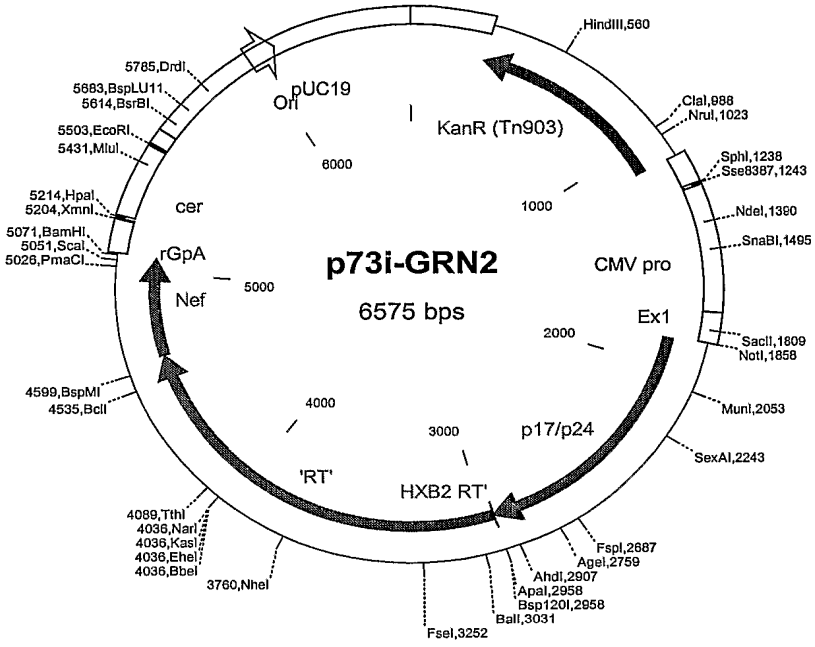
[SEQ ID NO: 69]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6575 bps
pUC19 Ori
KanR (Tn903)
CMV pro
Ex1
p17/p24
HXB2 RT'
'RT'
Nef
rGpA
cer
1000
2000
3000
4000
5000
6000
HindIII,560
ClaI,988
NruI,1023
SphI,1238
Sse8387,1243
NdeI,1390
SnaBI,1495
SacII,1809
NotI,1858
MunI,2053
SexAI,2243
FspI,2687
AgeI,2759
AhdI,2907
ApaI,2958
Bsp120I,2958
BalI,3031
FseI,3252
3760,NheI
4089,TthI
4036,NarI
4036,KasI
4036,EheI
4036,BbeI
4599,BspMI
4535,BclI
5214,HpaI
5204,XmnI
5071,BamHI
5051,ScaI
5026,PmaCI
5503,EcoRI
5431,MluI
5683,BspLU11
5614,BsrBI
5785,DrdI

Figure 12

Sequence of the p17/p24opt/trNef insert in p73i-GN2:

ATGGGTGCCCGAGCTTCGGTACTGTCTGGTGGAGAGCTGGACAGATGGGAGAAAATT
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CGCCAGATCCTGGGGCAATTGCAGCCATCCCTCCAGACCGGGAGTGAAGAGCTGAGG
TCCTTGTATAACACAGTGGCTACCCTCTACTGCGTACACCAGAGGATCGAGATTAAG
GATACCAAGGAGGCCTTGGACAAAATTGAGGAGGAGCAAAACAAGAGCAAGAAGAAG
GCCCAGCAGGCAGCTGCTGACACTGGGCATAGCAACCAGGTATCACAGAACTATCCT
ATTGTCCAAAACATTCAGGGCCAGATGGTTCATCAGGCCATCAGCCCCCGGACGCTC
AATGCCTGGGTGAAGGTTGTCGAAGAGAAGGCCTTTTCTCCTGAGGTTATCCCCATG
TTCTCCGCTTTGAGTGAGGGGGCCACTCCTCAGGACCTCAATACAATGCTTAATACC
GTGGGCGGCCATCAGGCCGCCATGCAAATGTTGAAGGAGACTATCAACGAGGAGGCA
GCCGAGTGGGACAGAGTGCATCCCGTCCACGCTGGCCCAATCGCGCCCGGACAGATG
CGGGAGCCTCGCGGCTCTGACATTGCCGGCACCACCTCTACACTGCAAGAGCAAATC
GGATGGATGACCAACAATCCTCCCATCCCAGTTGGAGAAATCTATAAACGGTGGATC
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AGACAGGGACCCAAAGAGCCTTTTAGGGATTACGTCGACCGGTTTTATAAGACCCTG
CGAGCAGAGCAGGCCTCTCAGGAGGTCAAAAACTGGATGACGGAGACACTCCTGGTA
CAGAACGCTAACCCCGACTGCAAAACAATCTTGAAGGCACTAGGCCCGGCTGCCACC
CTGGAAGAGATGATGACCGCCTGTCAGGGAGTAGGCGGACCCGGACACAAAGCCAGA
GTGTTGATGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATGACTTAC
AAGGCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGGGGACTGGAAGGGCTA
ATTCACTCCCAAAGAAGACAAGATATCCTTGATCTGTGGATCTACCACACACAAGGC
TACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCAGATATCCACTGACC
TTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAGGCCAAT
AAAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGGATGGATGACCCG
GAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCC**G**CCTAGCATTTCATCACGTGGCC
CGAGAGCTGCATCCGGAGTACTTCAAGAACTGCTGA [SEQ ID NO: 70]

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LETSEGCRQI LGQLQPSLQT GSEELRSLYN TVATLYCVHQ RIEIKDTKEA
LDKIEEEQNK SKKKAQQAAA DTGHSNQVSQ NYPIVQNIQG QMVHQAISPR
TLNAWVKVVE EKAFSPEVIP MFSALSEGAT PQDLNTMLNT VGGHQAAMQM
LKETINEEAA EWDRVHPVHA GPIAPGQMRE PRGSDIAGTT STLQEQIGWM
TNNPPIPVGE IYKRWIILGL NKIVRMYSPT SILDIRQGPK EPFRDYVDRF
YKTLRAEQAS QEVKNWMTET LLVQNANPDC KTILKALGPA ATLEEMMTAC
QGVGGPGHKA RVLMVGFPVT PQVPLRPMTY KAAVDLSHFL KEKGGLEGLI
HSQRRQDILD LWIYHTQGYF PDWQNYTPGP GVRYPLTFGW CYKLVPVEPD
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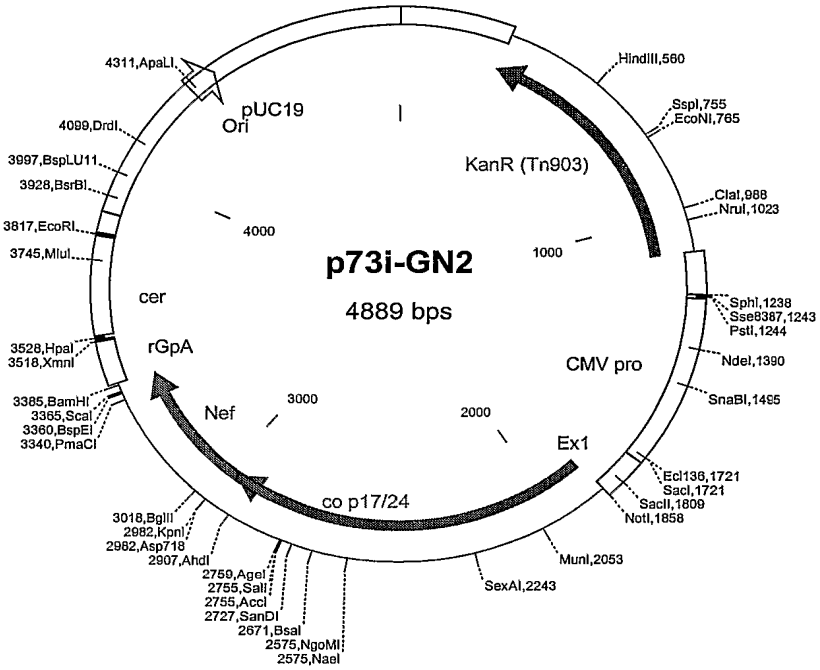
YFKNC* [SEQ ID NO: 71]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pUC19
Ori
KanR (Tn903)
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Ex1
co p17/24
Nef
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cer
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2000
3000
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4311,ApaLI
4099,DrdI
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3745,MluI
3528,HpaI
3518,XmnI
3385,BamHI
3365,ScaI
3360,BspEI
3340,PmaCI
3018,BglII
2982,KpnI
2982,Asp718
2907,AhdI
2759,AgeI
2755,SalI
2755,AccI
2727,SanDI
2671,BsaI
2575,NgoMI
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SexAI,2243
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NotI,1858
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SacI,1721
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PstI,1244
SphI,1238
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EcoNI,765
SspI,755
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Figure 13

Sequence of RT w229k:

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TTTGCCATCAAGAAGAAGGACAGCACCAAGTGGCGCAAGCTGGTGGATTTCCGGGAGCTGAA
TAAGCGGACCCAGGATTTCTGGGAGGTCCAGCTGGGCATCCCCCATCCGGCCGGCCTGAAGA
AGAAGAAGAGCGTGACCGTGCTGGACGTGGGCGACGCTTACTTCAGCGTCCCTCTGGACGAG
GACTTTAGAAAGTACACCGCCTTTACCATCCCATCTATCAACAACGAGACCCCTGGCATCAG
ATATCAGTACAACGTCCTCCCCCAGGGCTGGAAGGGCTCTCCCGCCATTTTCCAGAGCTCCA
TGACCAAGATCCTGGAGCCGTTTCGGAAGCAGAACCCCGATATCGTCATCTACCAGTACATG
GACGACCTGTACGTGGGCTCTGACCTGGAAATCGGGCAGCATCGCACGAAGATTGAGGAGCT
GAGGCAGCATCTGCTGAGATGGGGCCTGACCACTCCGGACAAGAAGCATCAGAAGGAGCCGC
CATTCCTgaaGATGGGCTACGAGCTCCATCCCGACAAGTGGACCGTGCAGCCTATCGTCCTC
CCCGAGAAGGACAGCTGGACCGTGAACGACATCCAGAAGCTGGTGGGCAAGCTCAACTGGGC
TAGCCAGATCTATCCCGGGATCAAGGTGCGCCAGCTCTGCAAGCTGCTGCGCGGCACCAAGG
CCCTGACCGAGGTGATTCCCCTCACGGAGGAAGCCGAGCTCGAGCTGGCTGAGAACCGGGAG
ATCCTGAAGGAGCCCGTGCACGGCGTGTACTATGACCCCTCCAAGGACCTGATCGCCGAAAT
CCAGAAGCAGGGCCAGGGGCAGTGGACATACCAGATTTACCAGGAGCCTTTCAAGAACCTCA
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GCCGTCCAGAAGATCACGACCGAGTCCATCGTGATCTGGGGGAAGACACCCAAGTTCAAGCT
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CCCATCGTGGGCGCGGAGACATTCTACGTGGACGGCGCGGCCAACCGCGAAACAAAGCTCGG
GAAGGCCGGGTACGTCACCAACCGGGGCCGCCAGAAGGTCGTCACCCTGACCGACACCACCA
ACCAGAAGACGGAGCTGCAGGCCATCTATCTCGCTCTCCAGGACTCCGGCCTGGAGGTGAAC
ATCGTGACGGACAGCCAGTACGCGCTGGGCATTATTCAGGCCCAGCCGGACCAGTCCGAGAG
CGAACTGGTGAACCAGATTATCGAGCAGCTGATCAAGAAAGAGAAGGTCTACCTCGCCTGGG

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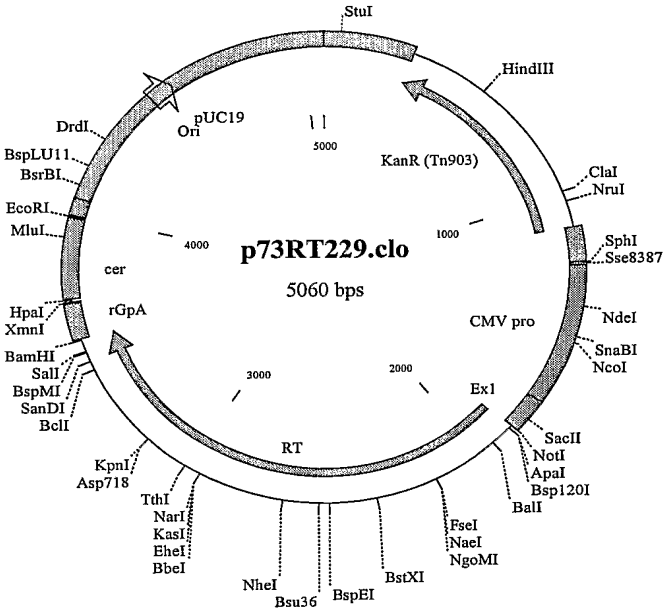
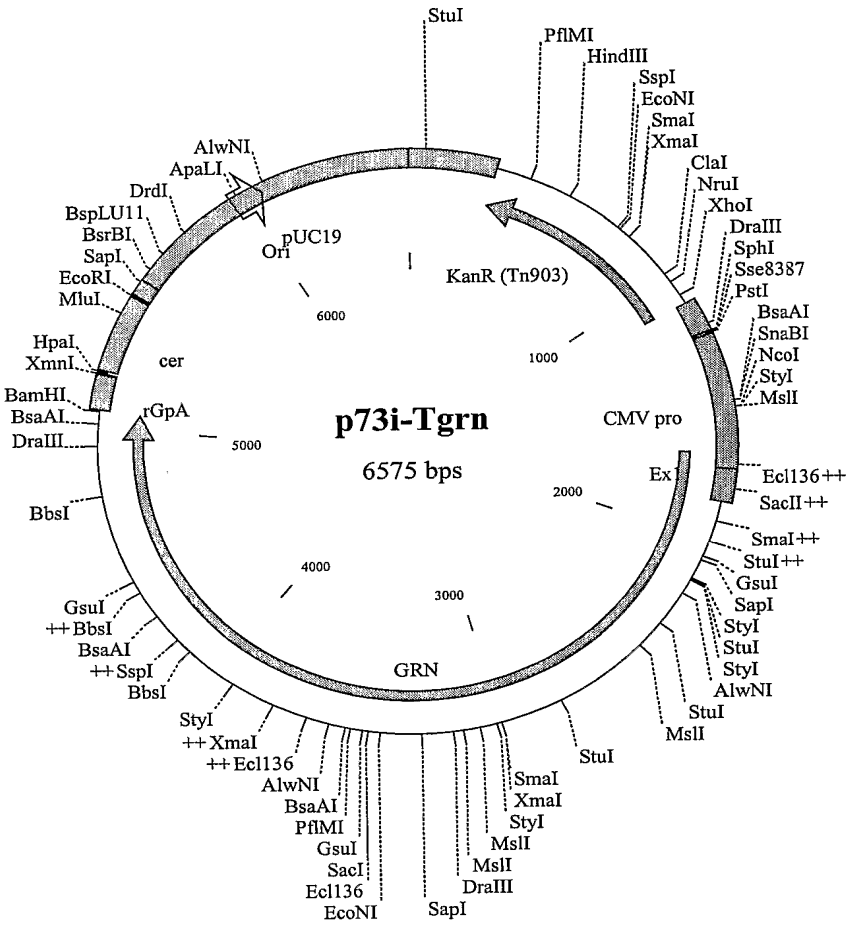


Figure 14



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TCCTTGTATAACACAGTGGCTACCCTCTACTGCGTACACCAGAGGATCGAGATTAAG
GATACCAAGGAGGCCTTGGACAAAATTGAGGAGGAGCAAAACAAGAGCAAGAAGAAG
GCCCAGCAGGCAGCTGCTGACACTGGGCATAGCAACCAGGTATCACAGAACTATCCT
ATTGTCCAAAACATTCAGGGCCAGATGGTTCATCAGGCCATCAGCCCCCGGACGCTC
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CCGGACCAGTCCGAGAGCGAACTGGTGAACCAGATTATCGAGCAGCTGATCAAGAAA
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GTCGACAAGCTGGTGAGTGCGGGGATTAGAAAGGTGCTGATGGTGGGTTTTCCAGTC
ACACCTCAGGTACCTTTAAGACCAATGACTTACAAGGCAGCTGTAGATCTTAGCCAC
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ATCCTTGATCTGTGGATCTACCACACACAAGGCTACTTCCCTGATTGGCAGAACTAC
ACACCAGGGCCAGGGGTCAGATATCCACTGACCTTTGGATGGTGCTACAAGCTAGTA
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LDKIEEEQNK SKKKAQQAAA DTGHSNQVSQ NYPIVQNIQG QMVHQAISPR
TLNAWVKVVE EKAFSPEVIP MFSALSEGAT PQDLNTMLNT VGGHQAAMQM
LKETINEEAA EWDRVHPVHA GPIAPGQMRE PRGSDIAGTT STLQEQIGWM
TNNPPIPVGE IYKRWIILGL NKIVRMYSPT SILDIRQGPK EPFRDYVDRF
YKTLRAEQAS QEVKNWMTET LLVQNANPDC KTILKALGPA ATLEEMMTAC
QGVGGPGHKA RVLMGPISPI ETVSVKLKPG MDGPKVKQWP LTEEKIKALV
EICTEMEKEG KISKIGPENP YNTPVFAIKK KDSTKWRKLV DFRELNKRTQ
DFWEVQLGIP HPAGLKKKKS VTVLDVGDAY FSVPLDEDFR KYTAFTIPSI
NNETPGIRYQ YNVLPQGWKG SPAIFQSSMT KILEPFRKQN PDIVIYQYMD
DLYVGSDLEI GQHRTKIEEL RQHLLRWGLT TPDKKHQKEP PFLWMGYELH
PDKWTVQPIV LPEKDSWTVN DIQKLVGKLN WASQIYPGIK VRQLCKLLRG
TKALTEVIPL TEEAELELAE NREILKEPVH GVYYDPSKDL IAEIQKQGQG
QWTYQIYQEP FKNLKTGKYA RMRGAHTNDV KQLTEAVQKI TTESIVIWGK
TPKFKLPIQK ETWETWWTEY WQATWIPEWE FVNTPPLVKL WYQLEKEPIV
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LQDSGLEVNI VTDSQYALGI IQAQPDQSES ELVNQIIEQL IKKEKVYLAW
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FLKEKGGLEG LIHSQRRQDI LDLWIYHTQG YFPDWQNYTP GPGVRYPLTF
GWCYKLVPVE PDKVEEANKG ENTSLLHPVS LHGMDDPERE VLEWRFDSRL
AFHHVARELH PEYFKNC*

[SEQ ID NO: 74]

Figure 15

Sequence of Tnrg:

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CCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCAGATATCCACTGACCTTTGGA
TGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAGGCCAATAAAGGA
GAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGGATGGATGACCCGGAGAGA
GAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCATTTCATCACGTGGCCCGAGAG
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LHPVSLHGMD DPEREVLEWR FDS**R**LAFHHV ARELHPEYFK NCMGPISPIE
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NTPVFAIKKK DSTKWRKLVD FRELNKRTQD FWEVQLGIPH PAGLKKKKSV
TVLDVGDAYF SVPLDEDFRK YTAFTIPSIN NETPGIRYQY NVLPQGWKGS
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RKVLMGARAS VLSGGELDRW EKIRLRPGGK KKYKLKHIVW ASRELERFAV
NPGLLETSEG CRQILGQLQP SLQTGSEELR SLYNTVATLY CVHQRIEIKD
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IGWMTNNPPI PVGEIYKRWI ILGLNKIVRM YSPTSILDIR QGPKEPFRDY
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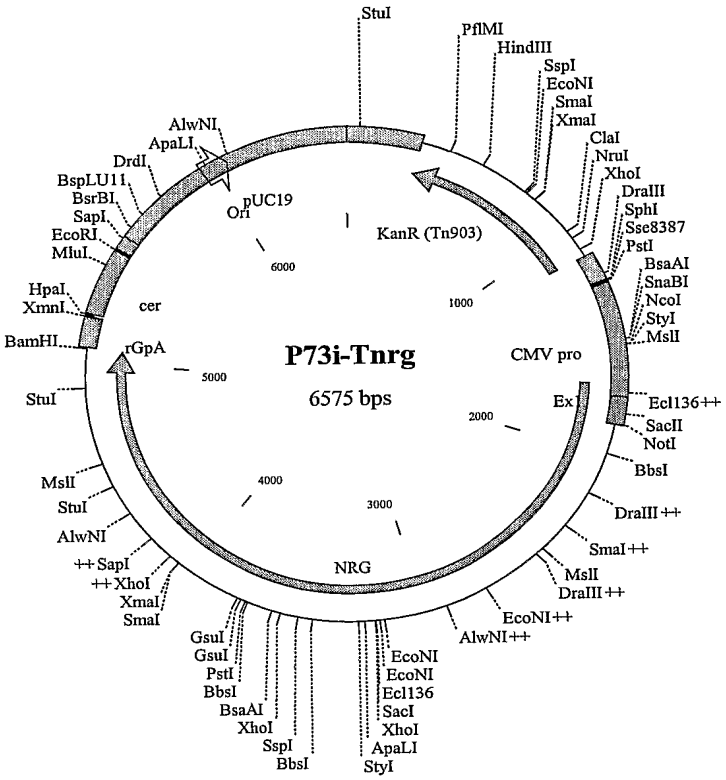

Figure 16

Sequence of the Tngr insert in p7313ie:

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CCGAGCTTCGGTACTGTCTGGTGGAGAGCTGGACAGATGGGAGAAAATTAGGCTGCGCCCGG
GAGGCAAAAAGAAATACAAGCTCAAGCATATCGTGTGGGCCTCGAGGGAGCTTGAACGGTTT
GCCGTGAACCCAGGCCTGCTGGAAACATCTGAGGGATGTCGCCAGATCCTGGGGCAATTGCA
GCCATCCCTCCAGACCGGGAGTGAAGAGCTGAGGTCCTTGTATAACACAGTGGCTACCCTCT
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QILGQLQPSL QTGSEELRSL YNTVATLYCV HQRIEIKDTK EALDKIEEEQ
NKSKKKAQQA AADTGHSNQV SQNYPIVQNI QGQMVHQAIS PRTLNAWVKV
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YQYNVLPQGW KGSPAIFQSS MTKILEPFRK QNPDIVIYQY MDDLYVGSDL
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IVLPEKDSWT VNDIQKLVGK LNWASQIYPG IKVRQLCKLL RGTKALTEVI
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QKETWETWWT EYWQATWIPE WEFVNTPPLV KLWYQLEKEP IVGAETFYVD
GAANRETKLG KAGYVTNRGR QKVVTLTDTT NQKTELQAIY LALQDSGLEV
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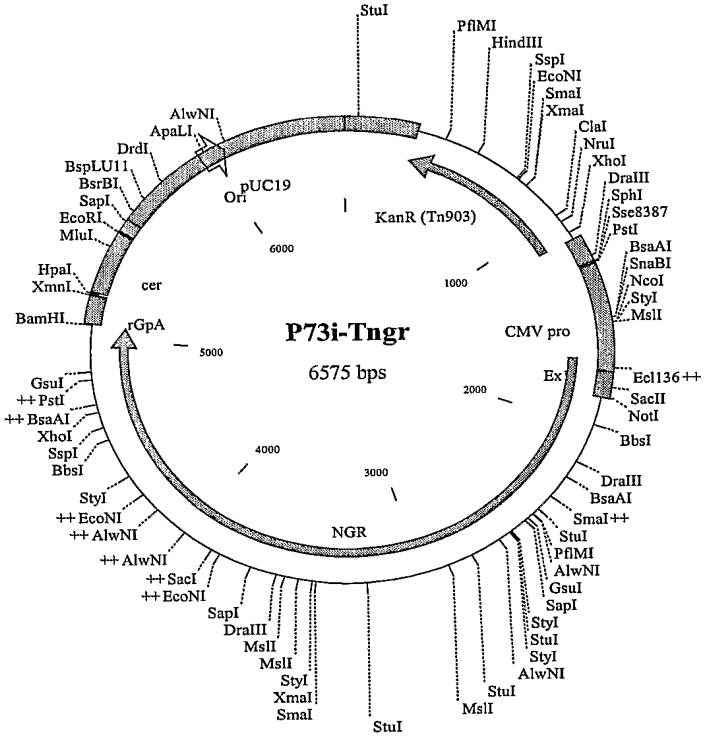
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6000
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PflMI
HindIII
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XmaI
ClaI
NruI
XhoI
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Sse8387
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SnaBI
NcoI
StyI
MslI
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BbsI
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XhoI
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++PstI
GsuI
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BsrBI
BspLU11
DrdI
ApaLI
AlwNI

Figure 17

Sequence of insert Trgn #6

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LPQGWKGSPA IFQSSMTKIL EPFRKQNPDI VIYQYMDDLY VGSDLEIGQH
RTKIEELRQH LLRWGLTTPD KKHQKEPPFL WMGYELHPDK WTVQPIVLPE
KDSWTVNDIQ KLVGKLNWAS QIYPGIKVRQ LCKLLRGTKA LTEVIPLTEE
AELELAENRE ILKEPVHGVY YDPSKDLIAE IQKQGQGQWT YQIYQEPFKN
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ETWWTEYWQA TWIPEWEFVN TPPLVKLWYQ LEKEPIVGAE TFYVDGAANR
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RELERFAVNP GLLETSEGCR QILGQLQPSL QTGSEELRSL YNTVATLYCV
HQRIEIKDTK EALDKIEEEQ NKSKKKAQQA AADTGHSNQV SQNYPIVQNI
QGQMVHQAIS PRTLNAWVKV VEEKAFSPEV IPMFSALSEG ATPQDLNTML
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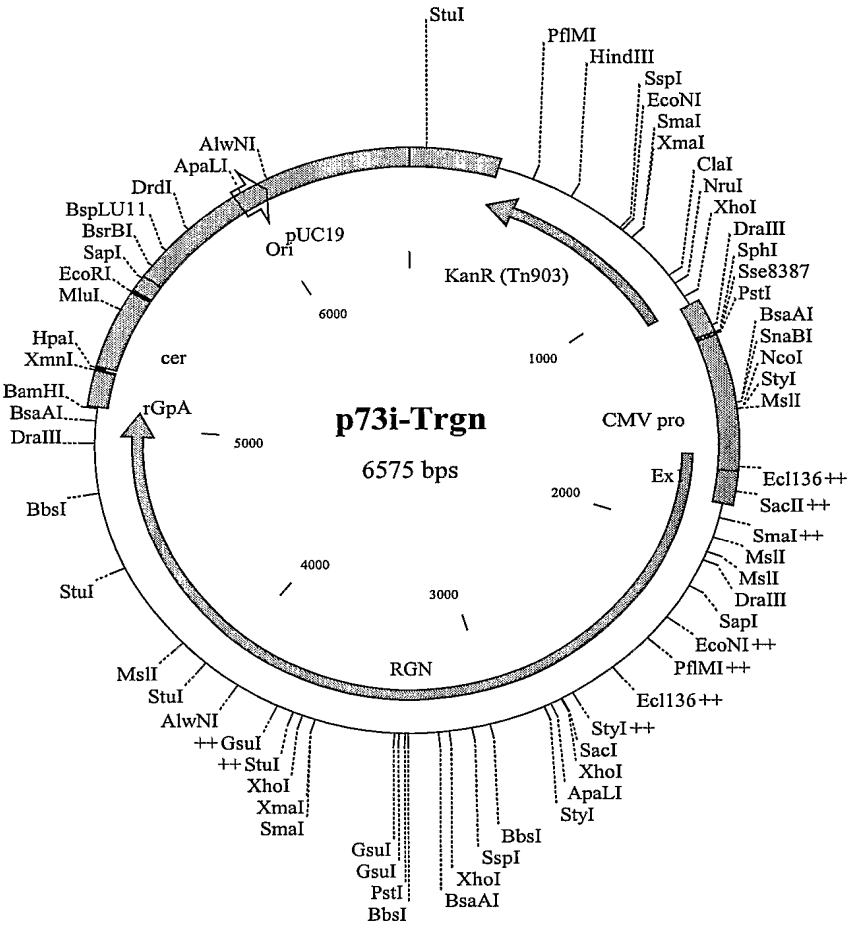

Figure 18

Sequence of insert of Trng #11

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LPQGWKGSPA IFQSSMTKIL EPFRKQNPDI VIYQYMDDLY VGSDLEIGQH
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AMQMLKETIN EEAAEWDRVH PVHAGPIAPG QMREPRGSDI AGTTSTLQEQ
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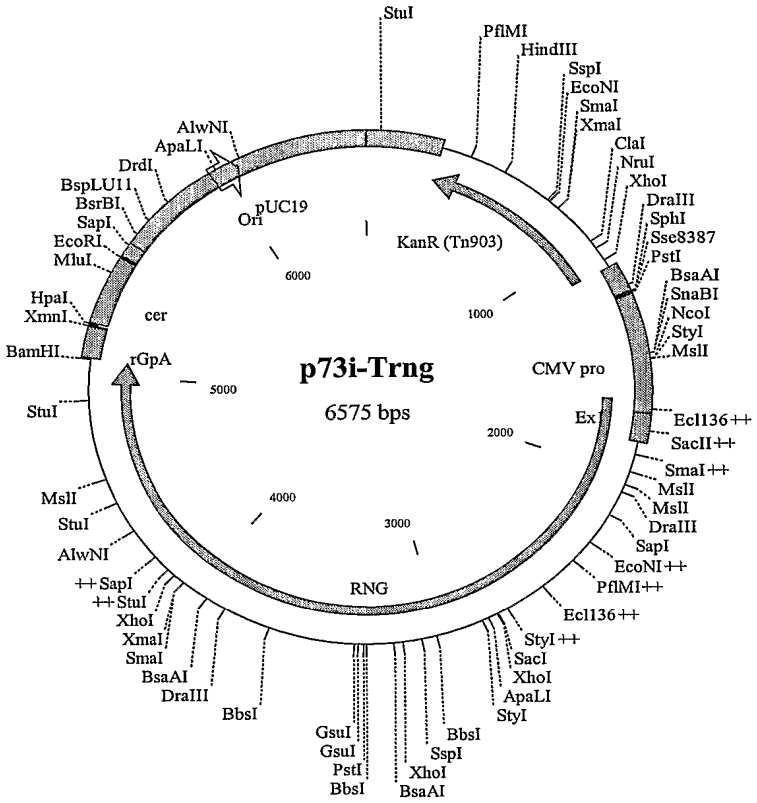
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SmaI
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ClaI
NruI
XhoI
DraIII
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++SapI
AlwNI
MslI
StuI
BamHI
HpaI
XmnI
EcoRI
MluI
SapI
BsrBI
BspLU11
DrdI
ApaLI
AlwNI

Figure 19

Sequence of TgnR (F1):

ATGGGTGCCCGAGCTTCGGTACTGTCTGGTGGAGAGCTGGACAGATGGGAGAAAATT
AGGCTGCGCCCGGGAGGCAAAAAGAAATACAAGCTCAAGCATATCGTGTGGGCCTCG
AGGGAGCTTGAACGGTTTGCCGTGAACCCAGGCCTGCTGGAAACATCTGAGGGATGT
CGCCAGATCCTGGGGCAATTGCAGCCATCCCTCCAGACCGGGAGTGAAGAGCTGAGG
TCCTTGTATAACACAGTGGCTACCCTCTACTGCGTACACCAGAGGATCGAGATTAAG
GATACCAAGGAGGCCTTGGACAAAATTGAGGAGGAGCAAAACAAGAGCAAGAAGAAG
GCCCAGCAGGCAGCTGCTGACACTGGGCATAGCAACCAGGTATCACAGAACTATCCT
ATTGTCCAAAACATTCAGGGCCAGATGGTTCATCAGGCCATCAGCCCCCGGACGCTC
AATGCCTGGGTGAAGGTTGTCGAAGAGAAGGCCTTTTCTCCTGAGGTTATCCCCATG
TTCTCCGCTTTGAGTGAGGGGGCCACTCCTCAGGACCTCAATACAATGCTTAATACC
GTGGGCGGCCATCAGGCCGCCATGCAAATGTTGAAGGAGACTATCAACGAGGAGGCA
GCCGAGTGGGACAGAGTGCATCCCGTCCACGCTGGCCCAATCGCGCCCGGACAGATG
CGGGAGCCTCGCGGCTCTGACATTGCCGGCACCACCTCTACACTGCAAGAGCAAATC
GGATGGATGACCAACAATCCTCCCATCCCAGTTGGAGAAATCTATAAACGGTGGATC
ATCCTGGGCCTGAACAAGATCGTGCGCATGTACTCTCCGACATCCATCCTTGACATT
AGACAGGGACCCAAAGAGCCTTTTAGGGATTACGTCGACCGGTTTTATAAGACCCTG
CGAGCAGAGCAGGCCTCTCAGGAGGTCAAAAACTGGATGACGGAGACACTCCTGGTA
CAGAACGCTAACCCCGACTGCAAAACAATCTTGAAGGCACTAGGCCCGGCTGCCACC
CTGGAAGAGATGATGACCGCCTGTCAGGGAGTAGGCGGACCCGGACACAAAGCCAGA
GTGTTGATGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATGACTTAC
AAGGCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGGGGACTGGAAGGGCTA
ATTCACTCCCAAAGAAGACAAGATATCCTTGATCTGTGGATCTACCACACACAAGGC
TACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCAGATATCCACTGACC
TTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAGGCCAAT
AAAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGGATGGATGACCCG
GAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCATTTCATCACGTGGCC
CGAGAGCTGCATCCGGAGTACTTCAAGAACTGCATGGGCCCCATCAGTCCCATCGAG
ACCGTGCCGGTGAAGCTGAAACCCGGGATGGACGGCCCCAAGGTCAAGCAGTGGCCA
CTCACCGAGGAGAAGATCAAGGCCCTGGTGGAGATCTGCACCGAGATGGAGAAAGAG
GGCAAGATCAGCAAGATCGGGCCTGAGAACCCATACAACACCCCCGTGTTTGCCATC
AAGAAGAAGGACAGCACCAAGTGGCGCAAGCTGGTGGATTTCCGGGAGCTGAATAAG
CGGACCCAGGATTTCTGGGAGGTCCAGCTGGGCATCCCCCATCCGGCCGGCCTGAAG
AAGAAGAAGAGCGTGACCGTGCTGGACGTGGGCGACGCTTACTTCAGCGTCCCTCTG
GACGAGGACTTTAGAAAGTACACCGCCTTTACCATCCCATCTATCAACAACGAGACC
CCTGGCATCAGATATCAGTACAACGTCCTCCCCCAGGGCTGGAAGGGCTCTCCCGCC
ATTTTCCAGAGCTCCATGACCAAGATCCTGGAGCCGTTTCGGAAGCAGAACCCCGAT
ATCGTCATCTACCAGTACATGGACGACCTGTACGTGGGCTCTGACCTGGAAATCGGG
CAGCATCGCACGAAGATTGAGGAGCTGAGGCAGCATCTGCTGAGATGGGGCCTGACC
ACTCCGGACAAGAAGCATCAGAAGGAGCCGCCATTCCTgaaGATGGGCTACGAGCTC
CATCCCGACAAGTGGACCGTGCAGCCTATCGTCCTCCCCGAGAAGGACAGCTGGACC
GTGAACGACATCCAGAAGCTGGTGGGCAAGCTCAACTGGGCTAGCCAGATCTATCCC
GGGATCAAGGTGCGCCAGCTCTGCAAGCTGCTGCGCGGCACCAAGGCCCTGACCGAG

GTGATTCCCCTCACGGAGGAAGCCGAGCTCGAGCTGGCTGAGAACCGGGAGATCCTG
AAGGAGCCCGTGCACGGCGTGTACTATGACCCCTCCAAGGACCTGATCGCCGAAATC
CAGAAGCAGGGCCAGGGGCAGTGGACATACCAGATTTACCAGGAGCCTTTCAAGAAC
CTCAAGACCGGCAAGTACGCCCGCATGAGGGGCGCCCACACCAACGATGTCAAGCAG
CTGACCGAGGCCGTCCAGAAGATCACGACCGAGTCCATCGTGATCTGGGGGAAGACA
CCCAAGTTCAAGCTGCCTATCCAGAAGGAGACCTGGGAGACGTGGTGGACCGAATAT
TGGCAGGCCACCTGGATTCCCGAGTGGGAGTTCGTGAATACACCTCCTCTGGTGAAG
CTGTGGTACCAGCTCGAGAAGGAGCCCATCGTGGGCGCGGAGACATTCTACGTGGAC
GGCGCGGCCAACCGCGAAACAAAGCTCGGGAAGGCCGGGTACGTCACCAACCGGGGC
CGCCAGAAGGTCGTCACCCTGACCGACACCACCAACCAGAAGACGGAGCTGCAGGCC
ATCTATCTCGCTCTCCAGGACTCCGGCCTGGAGGTGAACATCGTGACGGACAGCCAG
TACGCGCTGGGCATTATTCAGGCCCAGCCGGACCAGTCCGAGAGCGAACTGGTGAAC
CAGATTATCGAGCAGCTGATCAAGAAAGAGAAGGTCTACCTCGCCTGGGTCCCGGCC
CATAAGGGCATTGGCGGCAACGAGCAGGTCGACAAGCTGGTGAGTGCGGGGATTAGA
AAGGTGCTGTAA [SEQ ID NO: 83]

MGARASVLSG GELDRWEKIR LRPGGKKKYK LKHIVWASRE LERFAVNPGL
LETSEGCRQI LGQLQPSLQT GSEELRSLYN TVATLYCVHQ RIEIKDTKEA
LDKIEEEQNK SKKKAQQAAA DTGHSNQVSQ NYPIVQNIQG QMVHQAISPR
TLNAWVKVVE EKAFSPEVIP MFSALSEGAT PQDLNTMLNT VGGHQAAMQM
LKETINEEAA EWDRVHPVHA GPIAPGQMRE PRGSDIAGTT STLQEQIGWM
TNNPPIPVGE IYKRWIILGL NKIVRMYSPT SILDIRQGPK EPFRDYVDRF
YKTLRAEQAS QEVKNWMTET LLVQNANPDC KTILKALGPA ATLEEMMTAC
QGVGGPGHKA RVLMVGFPVT PQVPLRPMTY KAAVDLSHFL KEKGGLEGLI
HSQRRQDILD LWIYHTQGYF PDWQNYTPGP GVRYPLTFGW CYKLVPVEPD
KVEEANKGEN TSLLHPVSLH GMDDPEREVL EWRFDSRLAF HHVARELHPE
YFKNCMGPIS PIETVSVKLK PGMDGPKVKQ WPLTEEKIKA LVEICTEMEK
EGKISKIGPE NPYNTPVFAI KKKDSTKWRK LVDFRELNKR TQDFWEVQLG
IPHPAGLKKK KSVTVLDVGD AYFSVPLDED FRKYTAFTIP SINNETPGIR
YQYNVLPQGW KGSPAIFQSS MTKILEPFRK QNPDIVIYQY MDDLYVGSDL
EIGQHRTKIE ELRQHLLRWG LTTPDKKHQK EPPFLWMGYE LHPDKWTVQP
IVLPEKDSWT VNDIQKLVGK LNWASQIYPG IKVRQLCKLL RGTKALTEVI
PLTEEAELEL AENREILKEP VHGVYYDPSK DLIAEIQKQG QGQWTYQIYQ
EPFKNLKTGK YARMRGAHTN DVKQLTEAVQ KITTESIVIW GKTPKFKLPI
QKETWETWWT EYWQATWIPE WEFVNTPPLV KLWYQLEKEP IVGAETFYVD
GAANRETKLG KAGYVTNRGR QKVVTLTDTT NQKTELQAIY LALQDSGLEV
NIVTDSQYAL GIIQAQPDQS ESELVNQIIE QLIKKEKVYL AWVPAHKGIG
GNEQVDKLVS AGIRKVL*
[SEQ ID NO: 84]

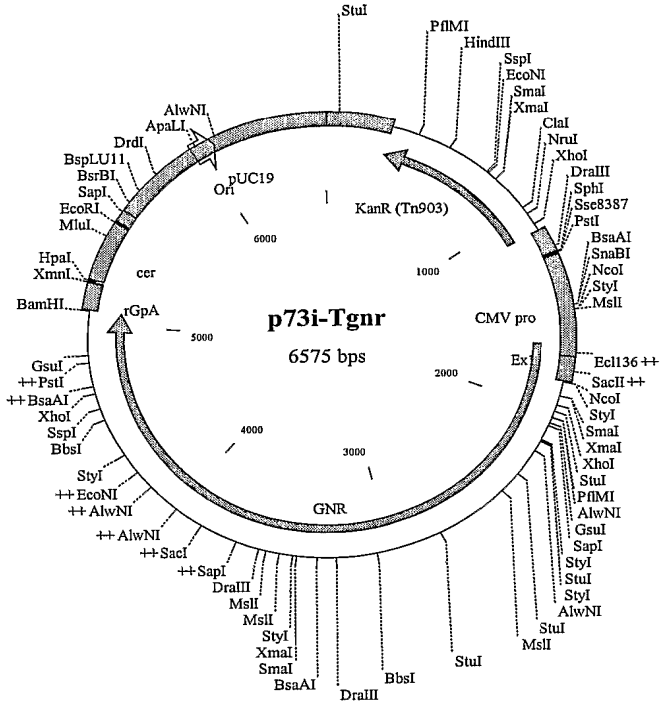

p73i-Tgnr
6575 bps
pUC19 Ori
KanR (Tn903)
CMV pro
GNR
rGpA
cer
1000
2000
3000
4000
5000
6000
StuI
PflMI
HindIII
SspI
EcoNI
SmaI
XmaI
ClaI
NruI
XhoI
DraIII
SphI
Sse8387
PstI
BsaAI
SnaBI
NcoI
StyI
MslI
Ecl136++
SacII++
NcoI
StyI
SmaI
XmaI
XhoI
StuI
PflMI
AlwNI
GsuI
SapI
StyI
StuI
StyI
AlwNI
StuI
MslI
StuI
BbsI
DraIII
BsaAI
SmaI
XmaI
StyI
MslI
MslI
DraIII
SapI++
SacI++
AlwNI++
AlwNI++
EcoNI++
StyI
BbsI
SspI
XhoI
BsaAI++
PstI++
GsuI
BamHI
HpaI
XmnI
MluI
EcoRI
SapI
BsrBI
BspLU11
DrdI
ApaLI
AlwNI

Figure 20.

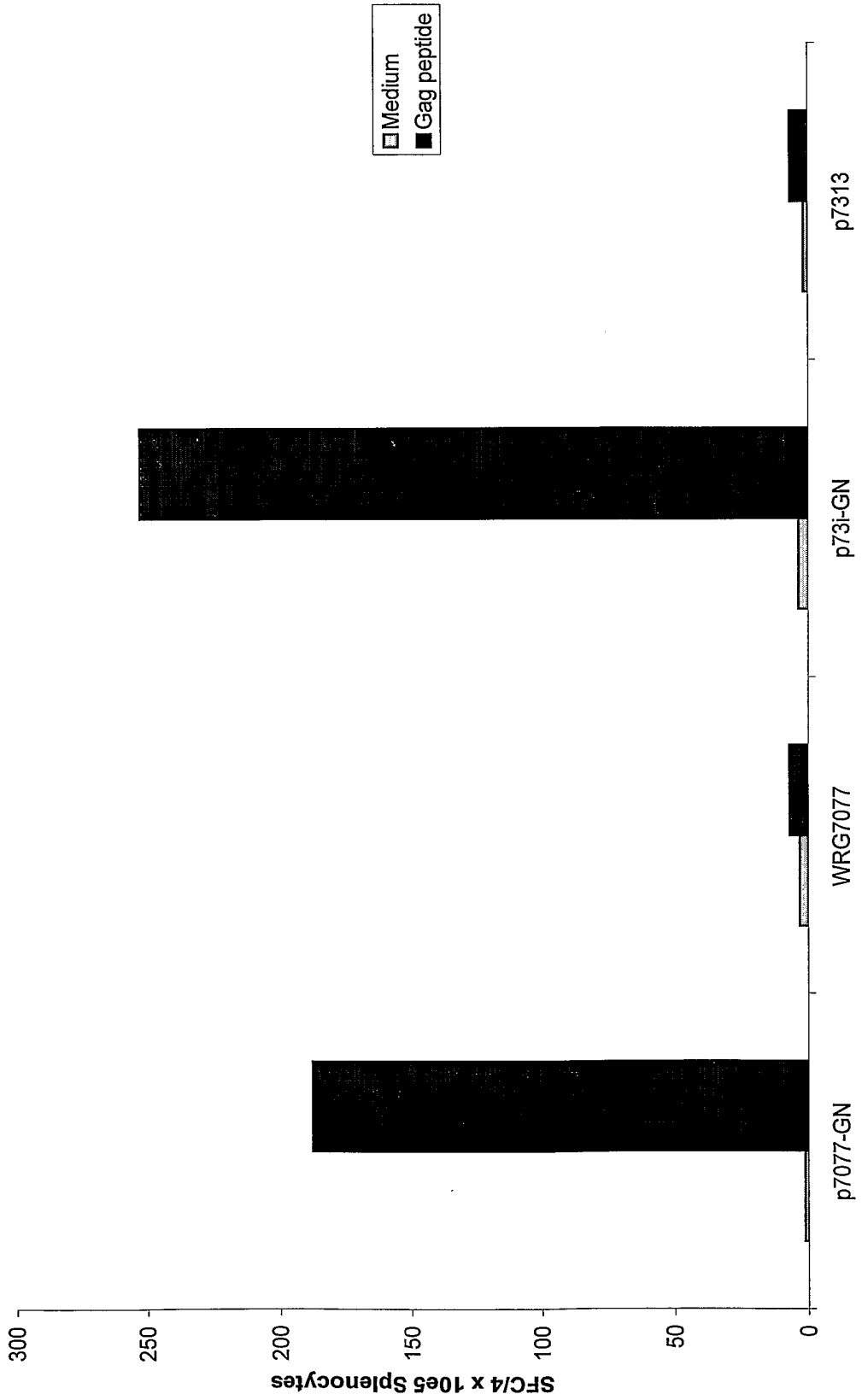
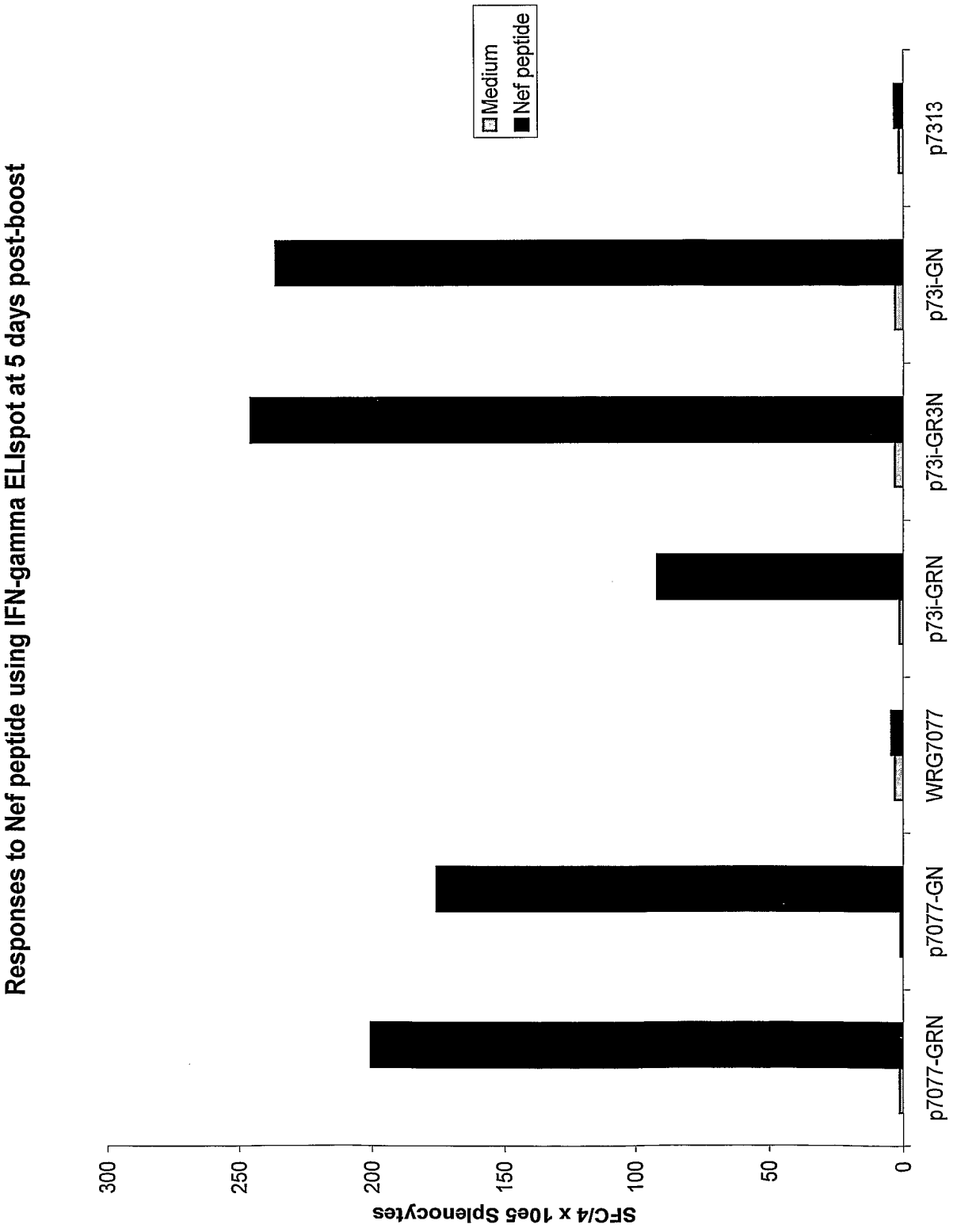

Responses to Gag peptide measured using IFN-gamma ELIspot at 5 days post-boost
Medium
Gag peptide
SFC/4 x 10e5 Splenocytes
300
250
200
150
100
50
0
p7077-GN
WRG7077
p73i-GN
p7313

Figure 21.


Responses to Nef peptide using IFN-gamma ELIspot at 5 days post-boost
Medium
Nef peptide
SFC/4 x 10e5 Splenocytes
300
250
200
150
100
50
0
p7077-GRN
p7077-GN
WRG7077
p73i-GRN
p73i-GR3N
p73i-GN
p7313

PG4653

Figure 22.

Responses to Rt peptide by IFN-gamma ELIspot at 5 days post-boost

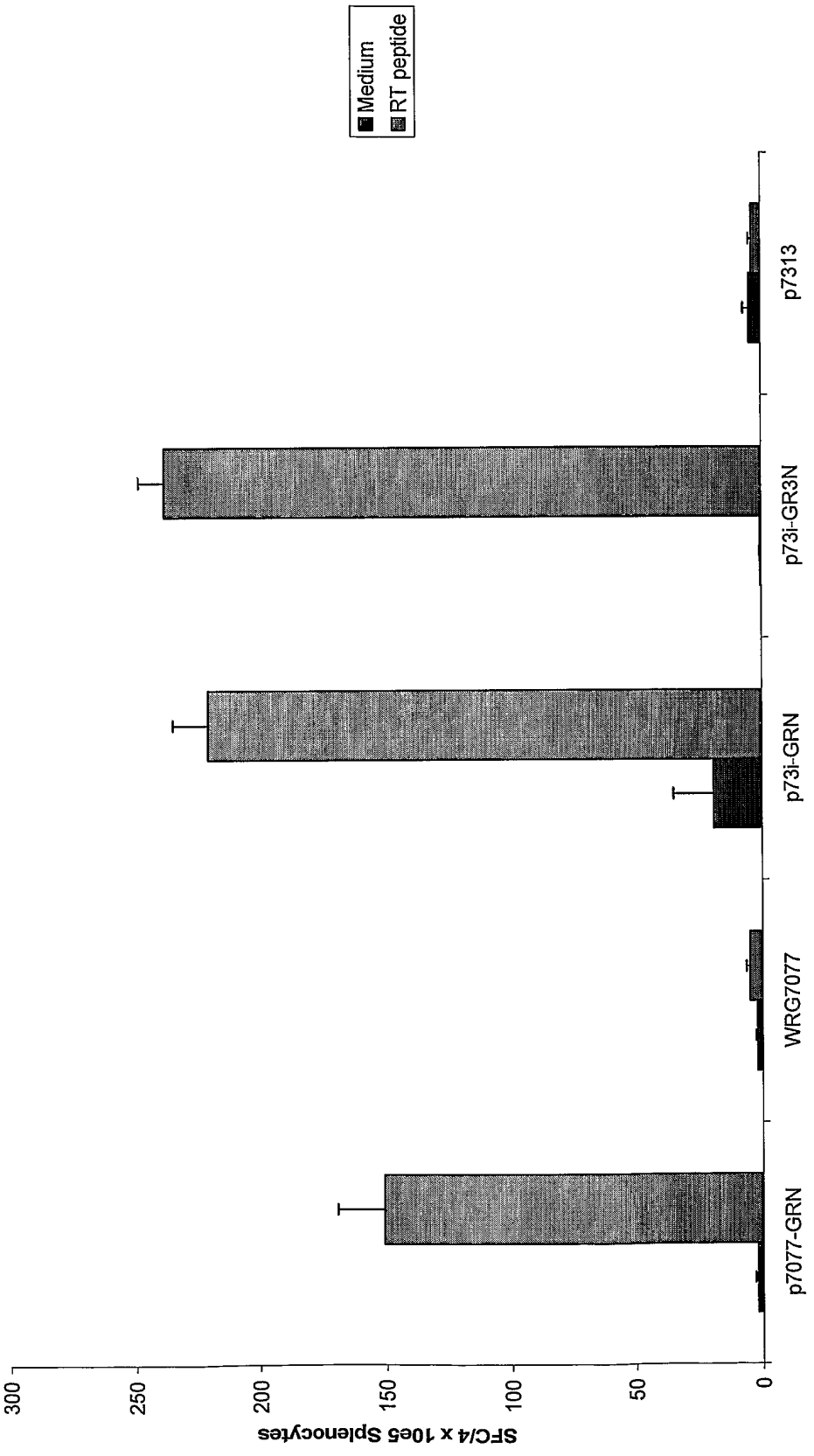
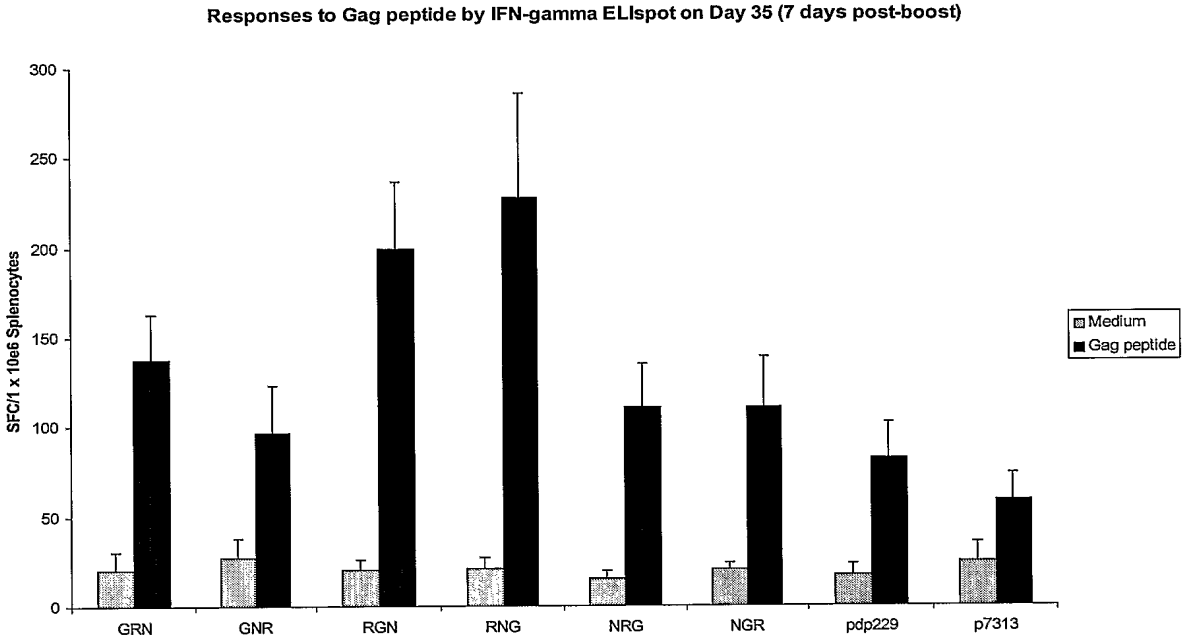
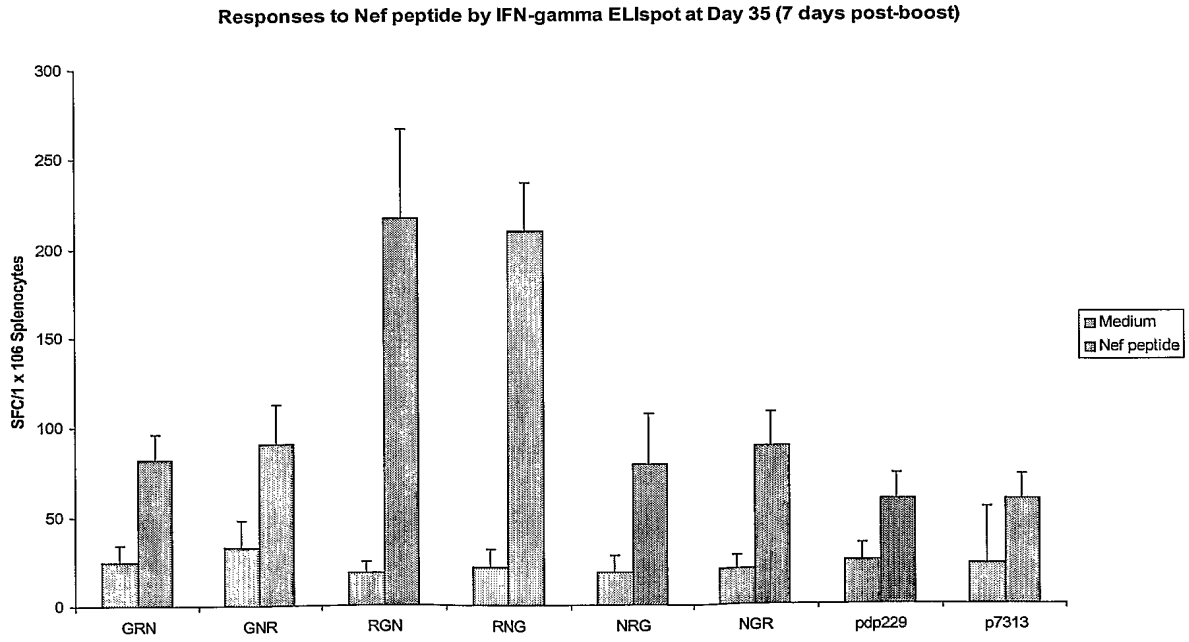


Figure 23

Example of the CD8 immune response that is detected in the mouse model following immunisation with the reformatted constructs. The constructs are comprised Gag, RT and Nef as a triple fusion. G is Gag, R is RT and N is Nef.





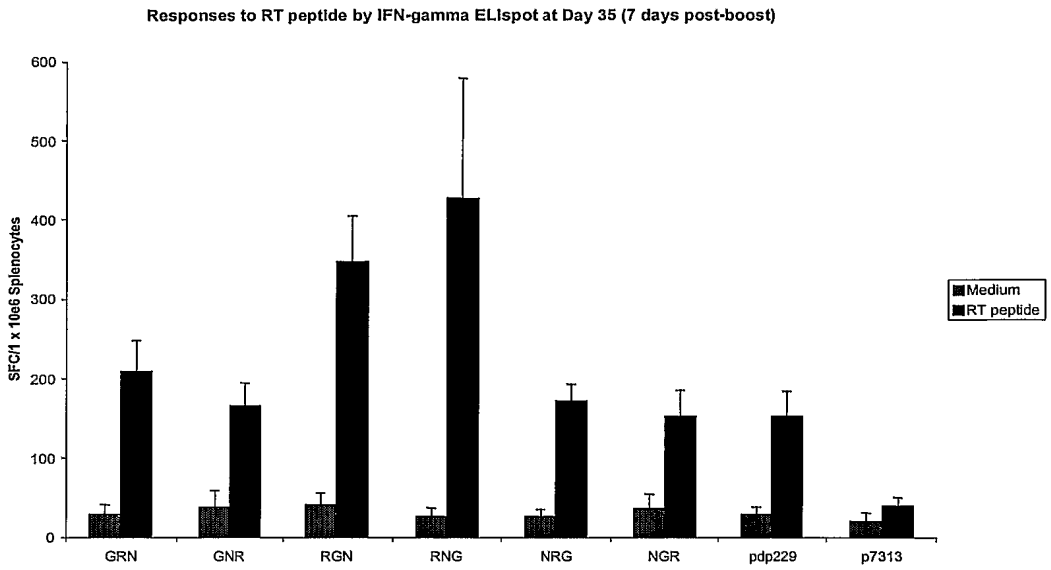
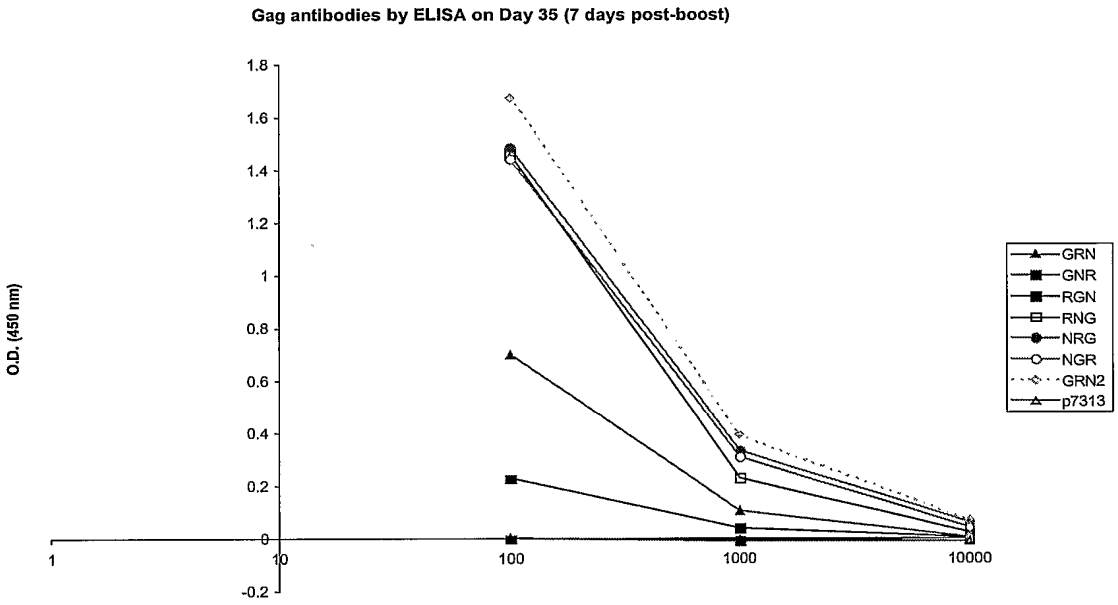

Responses to RT peptide by IFN-gamma ELIspot at Day 35 (7 days post-boost)
SFC/1 x 10e6 Splenocytes
600
500
400
300
200
100
0
GRN
GNR
RGN
RNG
NRG
NGR
pdp229
p7313
Medium
RT peptide

Figure 24


Gag antibodies by ELISA on Day 35 (7 days post-boost)
O.D. (450 nm)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
1
10
100
1000
10000
GRN
GNR
RGN
RNG
NRG
NGR
GRN2
p7313